Identification and characterization of three new human β-defensins: hBD23, hBD27, and hBD29

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Abstract

Human β-defensins are a family of cationic peptides which share a pattern of 6 conserved cysteine residues forming three disulfide bonds. Defensins are components of the innate immune system and are involved in proinflammatory processes. The present work describes the cloning and characterization of the cDNAs of three novel β-defensin genes (DEFB23, DEFB27, and DEFB29) identified by bioinformatical approach. The genes are clustered in two different contigs on chromosome 20. The genes consist of two exons and conserved exon-intron boundary regions. From cDNA analysis, secretory signal peptides and unusually long carboxy-terminal extensions following the β-defensin cysteine core were deduced. Expression analysis in 28 human tissues revealed the occurrence of the transcripts in only a few organs. The highest abundance was found in the male genital tract. A more detailed study in human epididymis was carried out. The expression of the new β-defensin genes, studied by real-time quantitative RT-PCR, is distinctively distributed along the functionally different segments of the epididymal duct. *In situ* hybridization on human epididymal tissue sections for DEFB29 provided evidence that the expression is restricted to the epithelial cell layer, which is known to secrete factors for sperm maturation. In addition, the immunodetection of hBD4, another recently discovered β-defensin, showed its location in the epithelial cells, possibly in their secretory vesicles as well as surrounding the sperm migrating within the epididymal lumen. β-defensins are considered as antimicrobial peptides. However, hBD27 did not exhibit antimicrobial activity against pathogenic and non-pathogenic germs. It appears that the peptides encoded by DEFB23, DEFB27, and DEFB29 genes exert physiological functions in the male genital tract, which may not be related to bacterial inhibition in host defense.

Keywords: β-defensin; Chromosome 20; Expression; Epididymis.

Zusammenfassung

Humane β-Defensine bilden eine Gruppe von kationischen Peptiden, die sich durch ein konserviertes Cysteinmotiv, welches aus 6 miteinander verbrückten Cysteinen besteht, auszeichnet. Defensine stellen einen wichtigen Bestandteil der angeborenen Immunabwehr dar und wirken regulatorisch in entzündungsfördernden Prozessen. Die vorliegende Arbeit beschreibt die Identifizierung und Charakterisierung von drei neuen humanen β-Defensin-Genen (DEFB23, DEFB27 und DEFB29) mit Hilfe eines bioinformatischen Ansatzes. Die identifizierten Gene sind in zwei verschiedenen Regionen auf Chromosom 20 lokalisiert. Sie bestehen aus zwei Exonen und einem konservierten Exon-Intron Übergang. cDNA-Analysen ergaben ein für sekretorische Peptide typisches Signalpeptid sowie eine ungewöhnlich lange dem Cysteinmotiv C-terminal folgende Aminosäuresequenz. Eine in 28 humanen Geweben durchgeführte Expressionsanalyse zeigte, dass DEFB23, DEFB27 und DEFB29 nur in wenigen Geweben transkribiert werden, wobei die höchste Expression im männlichem Genitaltrakt gefunden wurde. Ausführliche Untersuchungen in epididymalem Gewebe mit Hilfe von zeitaufgelöster quantitativer RT-PCR ergaben entlang dem Verlauf der Epididymis eine unterschiedliche Verteilung der neu entdeckten Defensin-Gentranskripte. *In situ* Hybridisierungs-Experimente an humanen epididymalen Gewebeschnitten mit cDNA-Sonden gegen die neuen β-Defensin-Transkripte zeigten darüber hinaus, dass die Expression von DEFB29 auf die epitheliale Zellschicht, welche zur Spermareifung notwendige Faktoren sekretiert, beschränkt ist. Weiterhin wurde mit Hilfe von Immunhistochemie die Lokalisation von hBD4, eines ebenfalls kürzlich identifizierten β-Defensins, entlang des männlichen Reproduktionstrakts nachgewiesen. Dabei wurde des weiteren festgestellt, dass die Immunreaktion gegen hBD4 sich vorwiegend in epithelialen Zellen und deren sekretorischen Vesikeln, sowie um wandernde Spermien herum, befindet. Obwohl β-Defensine als antimikrobielle Peptide aufgefasst werden, zeigte hBD27 keine antimikrobielle Aktivität gegen pathogene und nicht-pathogene Keime. Es scheint somit, dass die von DEFB23, DEFB27 und DEFB29 kodierten Peptide eine physiologische Funktion im männlichen Genitaltrakt erfüllen, die nicht durch die Hemmung mikrobiellen Wachstums erklärt werden kann.

Stichwörter: β-Defensin; Chromosom 20; Expression; Epididymis.

Abbreviations

ATCC	American Type Culture Collection
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
CT	Threshold cycle
Da	Dalton
DAPI	4',6-Diamidino-2-phenylindole
ddH_2O	Bidestilated water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Desoxyribonuclease
dNTP	Desoxynucleotidetriphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESTs	Expressed sequence tags
et al	et altera
EtOH	Ethanol
FCS	Fetal calf serum
g	Gravity
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
HPRT	Hypoxantine phosphoribosyltransferase
HRP	Horseradish-peroxidase
IL-1	Interleukin-1
LB	Luria-Bertani medium
LPS	Lipopolysaccharide
NBT	Nitro blue tetrazolium chloride
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PMA	Phorbol-12-myristate-13-acetate
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SSC	Standard saline citrate
TFA	Trifluoroacetic acid
TNF-α	Tumor necrosis factor α
Tris	Trishydroxymethyl-aminomethane buffer
TSB	Tryptic soy broth
U	Units
UV	Ultraviolet light
V	Voltage

Note: During the present thesis, the genes are cited as DEFB whereas the peptides are termed hBD.

Contents

1. Introduction

1.1 Cationic antimicrobial peptides

During the last decade, several cationic peptides have been discovered on the basis of their ability to inhibit the growth of microbial germs. Some antimicrobial peptides participate in the innate immune response by providing a rapid first-line defense against infection. Recent advances in this field have shown that peptides belonging to defensin and cathelicidin gene families are of particular importance for the mammalian immune defense system.

Cathelicidins were primarily identified in mammalian myeloid cells (ZANETTI *et al*., 1995). They are stored in the cytoplasmic granules of neutrophil leukocytes which release the antimicrobial peptides upon leukocyte activation. About 20 cathelicidin members have been identified in mammals (ZANETTI *et al*., 2001) but only one in humans (LARRICK *et al*., 1995). Cleavage of human cathelicidin (hCAP18) liberates its C-terminal, antimicrobial domain, a peptide named LL-37 because it begins with two leucine residues and is 37 amino acid residues in length. Human cathelicidin is often referred to as LL-37 (GUDMUNDSSON *et al*., 1996).

The first human α-defensins were named human neutrophil peptides (HNP) and originally identified as broad spectrum antimicrobial peptides (GANZ *et al*., 1985). As part of a study to define antimicrobial host defense of the mammalian airway, a basic, cysteine-rich peptide expressed in the bovine tracheal epithelium was discovered (DIAMOND *et al*., 1991). This molecule, tracheal antimicrobial peptide (TAP), exhibited numerous similarities to the α-defensins, but did not maintain the strict α-defensin cysteine motif. Shortly thereafter, 13 new peptides were discovered in bovine neutrophils, which also showed the same six-cysteine motif as TAP (SELSTED *et al*., 1993). These discoveries resulted in the creation of a second class of defensins called β-defensins.

Mammalian defensins are a family of secreted cationic and highly disulfide-bonded peptides which are considered as antimicrobial agents involved in host defense. Antimicrobial peptides can be divided into numerous categories based on their primary and secondary structures but most of them maintain certain common structural characteristics. Based on the spatial distribution of the cysteine residues and the disulfide bridges, the vertebrate defensins are subdivided into α-, β- and θ defensins.

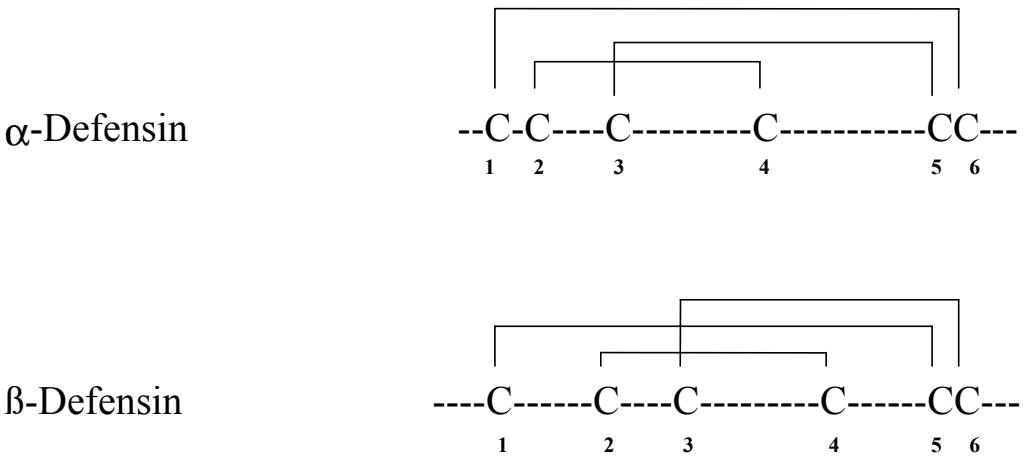


Figure 1. Comparison of disulfide bridges and the six-cysteine pattern of α- and β- defensins.
α-and β-defensins differ in the location and position of the cysteine residues and in their disulfide motifs. Disulfide linkages of the bridges for α-defensins (1-6, 2-4, 3-5), and for β-defensins (1-5, 2-4, and 3-6) are shown.

In the case of θ-defensins, the peptide termed rhesus theta defensin-1 (RTD-1) is a cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. This cyclic peptide has six cysteine residues linking: C1 to C6, C2 to C5 and C3 to C4 (TANG *et al*., 1999). RTD-2 and RTD-3 were recently isolated from circulating *Rhesus macaque* leukocytes (TRAN *et al*., 2002).

Six human α-defensins are presently known. Human neutrophil defensins are small (3.5-4 kDa) cationic peptides (GANZ *et al*., 1985). They are present in large amounts in azurophilic granules and released upon neutrophil activation (GANZ *et al.,* 1990). Four of these homologue members of the neutrophil defensins (HNP1 to HNP4) have been identified. Subsequent studies have shown that neutrophil defensins also display cytotoxic activity to eukaryotic cells and a variety of other pro- and anti-inflammatory activities (VAN WETERING *et al.,* 1999). HNP1, HNP2, and HNP3 might be related to the anti-HIV-1 activity of CAF (a soluble factor that suppresses HIV-1 replication) (ZHANG *et al*., 2002). The remaining α-defensins are HD5 and HD6. They were found to be expressed in small intestinal Paneth cells (specialized secretory cells at the base of the small intestinal crypts), as well as in epithelial cells of the female genital tract and are known as mediators of antimicrobial defense (QUAYLE *et al*., 1998). In mouse, Paneth cells release granules into the lumen of the crypts of Lieberkuhn in the small intestine, where the α-defensins (cryptdins) participate in mucosal immunity. Mouse cryptdins 1-6 are potent antimicrobial agents against certain microorganisms (OUELLETTE *et al*., 1992; OUELLETTE *et al*., 1997; EISENHAUER *et al*., 1992).

To date, six human β-defensins have been described. The first human β-defensin (hBD1) was originally isolated from hemofiltrate (BENSCH *et al*., 1995). Later on, hBD2 (HARDER *et al*., 1997) and hBD3 (HARDER *et al*., 2001) were purified from skin. The genes DEFB4 (GARCIA *et al*., 2001-B), DEFB5, and DEFB6 (YAMAGUCHI *et al*., 2002) were recently described.

In addition, three human β-defensin-like peptides: HE2D, HE2C and HE2E, sharing the characteristic cysteine pattern of β-defensins, were identified (VON HORSTEN *et al.,* 2002). In mouse, six β-defensins are known: Defb1 to Defb6 (HUTTNER *et al*., 1997; MORRISON *et al*., 1999; BALS *et al*., 1999; JIA *et al*., 2000; YAMAGUCHI *et al*., 2001).

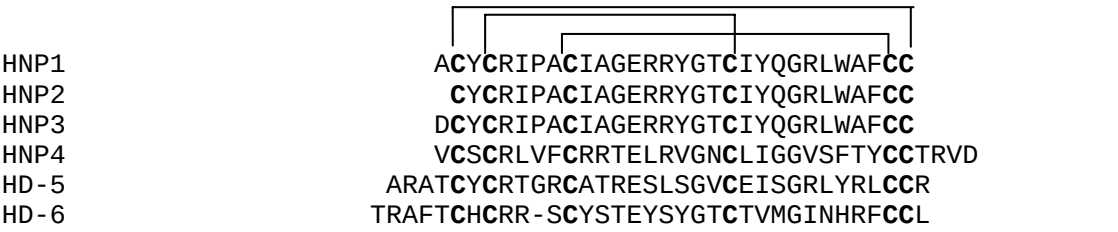
Defensins have also been described in other species (Table 1):

Table 1. β-defensins described in other species

Species	defensin	Authors
Monkey	θ-defensin (RTD-1)	TANG *et al*., 1999; TRAN *et al*., 2002
Equine	α-defensin (NAP-1)	COUTO *et al*., 1992
Bovine	α-defensin (BNBD1-13)	SELSTED *et al*., 1993
Bovine	β-defensin (TAP)	DIAMOND *et al*., 1996
Bovine	β-defensin (LAP)	RUSSEL *et al*., 1996
Bovine	β-defensin (EBD)	YOUNT *et al*., 1999
Porcine	β-defensin (pBD-1)	ZHANG *et al*., 1998; Shi *et al*., 1999
Sheep	β-defensin (sBD1-2)	HUTTNER *et al*., 1998
Chicken	β-defensin (Gal-3)	HARWIG *et al*., 1994
Rabbit	α-defensin (NP-1)	RAO *et al*., 1992
Rat	β-defensin (RBD1)	EISENHAUER *et al*., 1989
Plant	Plant-defensin (PDF1)	EPPLE *et al*., 1997
Insect	Insect-defensin	DIMARCQ *et al*., 1994; LEMAITRE *et al*., 1997; LEHRER *et al*., 1999

The amino acid sequences of human defensins and their disulfide bridges:

α-defensins



β-defensins

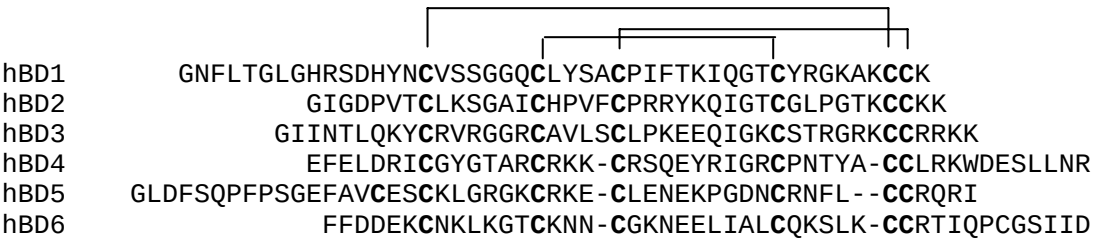


Figure 2. Primary amino acid sequences for the α- and β-defensins.
Bold letters indicate the conserved cysteine residues. The predicted disulfide linkages of these bridges for α-defensins are 1-6, 2-4, 3-5, whereas the disulfide linkages of the bridges for β-defensins are 1-5, 2-4, and 3-6.

Defensins have been predominantly considered to act as antimicrobial peptides forming an early barrier against pathogenic bacteria (SELSTED *et al*., 1984), fungi (GANZ *et al*., 1985) and viruses (LEHRER *et al*., 1985; ZHANG *et al*., 2002). Antimicrobial cationic peptides are an important component of the innate host defense. Antimicrobial peptides can also act in synergy with host molecules such as other cationic peptides or proteins, and lysozymes, as well as with conventional antibiotics. Innate immunity plays an important role in the host´s ability to avoid the progression of a potential pathogenic infection. In the last decade, the role of cationic peptides in antimicrobial defenses has become increasingly apparent, and there is a growing body of evidence that their role in defense against microbes may be as important to the host as antibodies, immune cells and phagocytes. In *Drosophila*, cationic antimicrobial peptides are well recognized as the major form of defense against infection and are induced, in response to challenge, by microbes or microbial-signaling molecules (DIMARCQ *et al*., 1994). In amphibians, crustaceans, fish, birds and mammals, it is becoming increasingly clear that antimicrobial peptides play a major role in local innate immunity, especially at mucosal and epithelial surfaces (ANDREU and RIVAS, 1998; HANCOCK and LEHRER, 1998). Defensins are the most widely studied family of antimicrobial peptides (COLE and GANZ, 2000). Most antimicrobial peptides have structures that are predominantly either α-helical or β-sheet. Structure-function studies of α-helical antimicrobial peptides have received considerable attention, partly due to their facile

chemical synthesis. Less is known about antimicrobial or β-sheet peptides that, like defensins, are challenging to be synthesized chemically.

1.2 Antimicrobial mechanism of action

Bacteria can be divided into two major groups, called Gram-positive and Gram-negative. The original distinction between Gram-positive and Gram-negative was based on a spatial staining procedure, the Gram stain, resulting from differences in cell wall structure. The Gram-negative cell wall is a multilayered structure and quite complex, while the Gram-positive cell wall consists of primarily a single type of molecule and is often much thicker. The activity and selectivity of cationic peptides are determined by their mode of interaction with bacterial cell membranes. For Gram-positive bacteria, a single cytoplasmic membrane and peptidoglycan is involved and is easier to disrupt than Gram-negative bacteria which comprise a more complex dual-membrane interaction. Like most antimicrobial peptides, defensins are cationic (polar) molecules with a positive charge due to arginine and lysine residues with spatially separated hydrophobic regions. This arrangement allows them to insert themselves into phospholipid membranes so that their cationic regions interact with anionic phospholipid head groups and water and their hydrophobic regions are buried within the hydrophobic membrane interior. In the membrane they can assemble into multimeric pores. Defensins and other antimicrobial peptides preferentially disrupt bacterial membranes that are rich in negatively charged phospholipids (HANCOCK *et al*., 1998). The killing event, for both Gram-positive and Gram-negative bacteria, is the formation of channels in the cytoplasmic membrane (Figure 3).

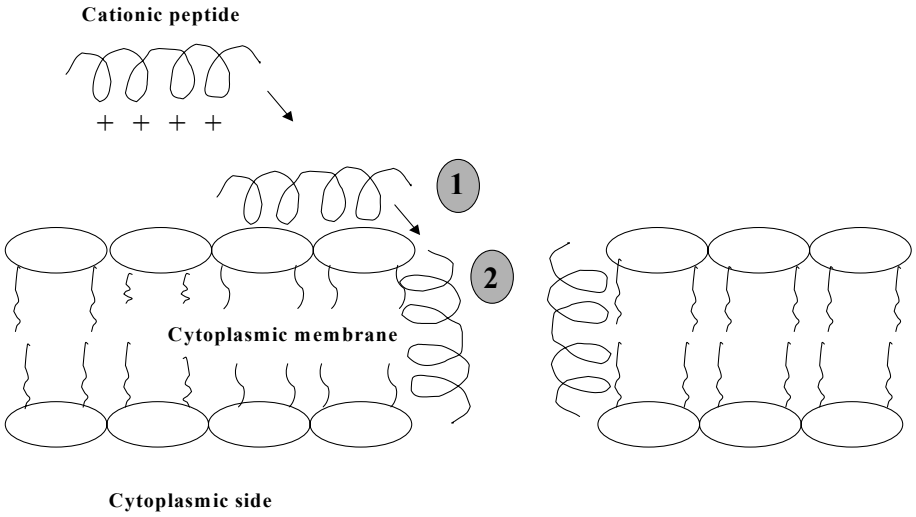


Figure 3. General mechanism of bacteria killing by cationic peptides.
Positively charged peptides bind to the external surface of negatively charged phospholipid bilayer (cytoplasmic membrane [1]). Then, cationic peptides form channels after localization of thinness on the bilayer (under the influence of membrane potential peptides insert into membrane) (2), leading to leakage of cytoplasmic molecules and cell death.

Gram-negative bacteria are formed by the outer membrane (Lipopolysaccharide, [LPS], proteins, phospholipids), the periplasm (lipoproteins and peptidoglycan) and additionally by the cytoplasmic membrane. Cationic peptides interact with LPS on the surface of Gram-negative bacteria. Because the peptides have a higher affinity for LPS than the native divalent cations Ca^{2+} or Mg^{2+}, they competitively displace these ions (HANCOCK *et al*., 1998). This effect causes a disruption to the normal barrier property of the outer membrane (Figure 4). Cationic peptides are thereby taken up across the outer membrane by a process termed self-promoted uptake (HANCOCK *et al*., 1998). It is thought that the affected membrane allows passage of a variety of molecules, including other antimicrobial and hydrophobic compounds, and facilitates the action of the perturbing peptide itself. This fact would explain their synergy with conventional antibiotics.

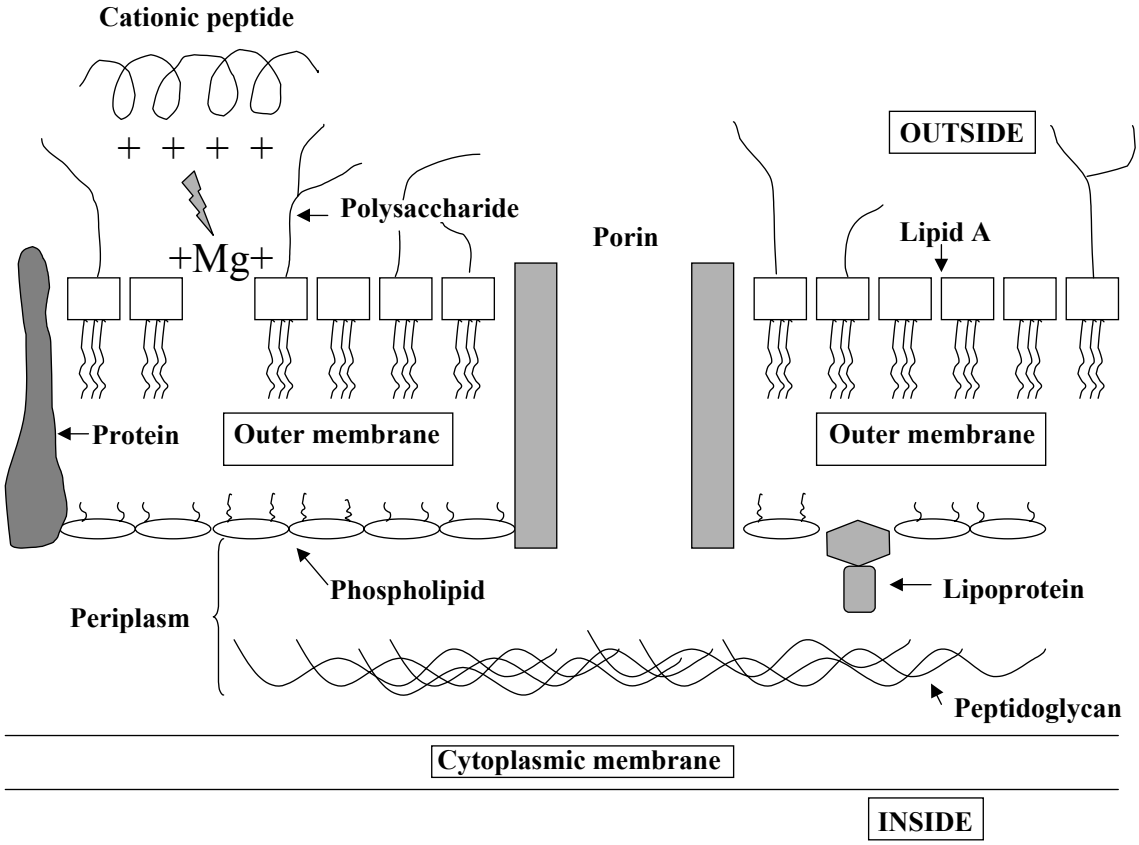


Figure 4. Arrangement of the different components in Gram-negative bacteria. Self-promoted uptake of antimicrobial cationic peptides across outer membranes of Gram-negative bacteria.
Positively charged peptides interact with negatively charged divalent-cation-binding sites on surface LPS, disrupting these sites and allowing the cationic peptides to pass across the outer membrane.

Other mechanisms of action enhance the membrane permeability, by the formation of a barrel stave pore (BOHEIM *et al*., 1994) or by a carpet mechanism (HE *et al*., 1996).

The lower anionic phospholipid content of the cell membranes of higher animals may provide relative protection from collateral damage produced by antimicrobial peptides. Nevertheless, cytotoxic effects in mammalian cells have also been reported (LEHRER *et al*., 1993).

1.3 Other functions of defensins

Cationic antimicrobial peptides are well known for their ability to kill many infective agents. However, the action of cationic antimicrobial peptides does not appear to be limited to the direct killing of microbes. There is accumulating evidence that they have an impressive variety of additional activities that can be expected to impact on the quality and effectiveness of innate immune responses and inflammation. These include many of the elements of inflammatory responses that are frequently ascribed to other agents. In fact, cationic antimicrobial peptides have been reported to be involved in many aspects of innate host defenses associated with acute inflammation. Some roles that cationic antimicrobial peptides may play in this process are based on their *in vitro* properties. Extracellular defensins, such as hBD2, exhibit chemotactic activity for immature dendritic cells (YANG *et al*., 1999), for recruitment of monocytes (TERRITO *et al*., 1989) and T-cells (CHERTOV *et al*., 1996). Defensins can also produce an enhancement of chemokine production and proliferative response of T helper cells (LILLARD *et al*., 1999), induction of specific macrophages and activated lymphocytes (LINDEMANN *et al*., 1994), induction of IL-8 production (VAN WETERING *et al*., 1997), regulation of complement activation (PANYUTICH *et al*., 1994), enhancing antigen-specific immunity (LILLARD *et al*., 1999; TANI *et al*., 2000), and an inhibitory effect of neutrophil defensins on tissue-type plasminogen activator (tPA)-mediated fibrinolysis (HIGAZI *et al*., 1996). These functions can be seen as a combined host defense system by linking direct bactericidal activity with recruitment of inflammatory cells and wound healing (VAN WETERING *et al*., 1999). Defensins are also involved in the inhibition of glucocorticoid production (SOLOMON *et al*., 1991), suppressors of ACTH activity on the mammalian adrenal cortex (ZHU *et al*., 1987), and are mitogenic for fibroblasts (MURPHY *et al*., 1993).

1.4 Functional genomics and mammalian defensin genes

The evolution of medical treatments has made remarkable strides aided by advances in the technology used for drug discovery. In the early days of pharmacology, therapeutic drugs were created from derivatives of medicinal plants. This was the case for aspirin, which was derived from the bark of the willow tree (ARONSON, 1994). Some beneficial drugs have been discovered

incidentally during scientific research. Over the last decade, the process of drug discovery has become more scientific and rational. It was during this period that many of the useful drugs that are currently still prescribed were discovered (e.g. calcium antagonists).

As researchers raced to complete the sequencing of the human genome, functional genomics became increasingly important for the success of genomics-based drug discovery and developmental efforts. Especially in the light of the recent description of the entire human genome which showed that humans possess almost twice as many genes as *Drosophila melanogaster*. It is not enough to know sequence information in order to utilize genomics to develop breakthrough drugs and diagnostics. One must also understand how genes and their products interact in pathways within the cell or the organism, and what roles they play in health and disease. The discovery of some substances or drugs was a process that moved from functional activity, through repeated screenings, towards an understanding of gene and molecular structure. Using current technology and genomics, scientists can now move in the opposite direction to that used in classical and traditional pharmacological research for new drug agents. This technique, referred to as functional genomics, has many benefits. The drug discovery process begins with genomic study, proceeds through screenings and ends with functional studies to provide putative drug candidates (Figure 5).

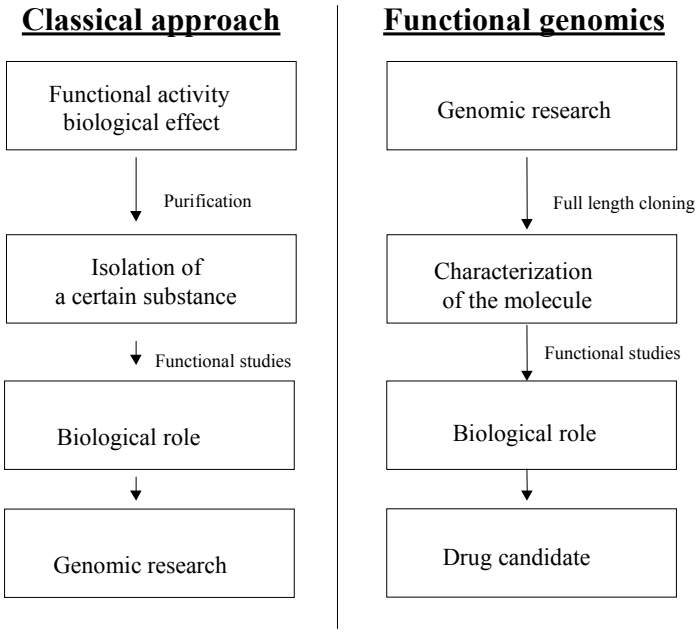


Figure 5. Comparison of the classical and functional genomic approaches in the drug discovery process.
The functional genomics approach differs from the classical approach especially in the initial and final stages of the scientific strategy.

The genome is defined as the complete set of genes in an organism. Genomics is of increasing interest to world leaders and was one of the topics discussed at the summit of industrialized nations held in Okinawa in June 2000. One of the greatest challenges currently facing pharmaceutical companies is to assimilate the vast amount of information generated by genomic and proteomic research into drug discovery programs.

As a result, drug discovery researchers are moving into a "post-genomic", functional genomics era. Functional genomics encompasses many traditional molecular genetics and other modern biological approaches combined with statistical and computational analysis of the results. The data produced by thousands of research teams all over the world are collected and organized in databases specialized for particular subjects. The fundamental strategy in a functional genomics approach is to expand the scope of biological investigation from studying single genes or proteins to studying all genes or proteins at once in a systematic fashion. As a consequence of the large amount of data produced in the field of molecular biology, most of the current bioinformatics projects deal with structural and functional aspects of genes and proteins. A technically challenging first priority is to generate complete sets of full-length cDNA clones and sequences for human and model-organism genes. Other functional genomics goals include studies of gene expression and regulation. Functional genomics, based on structural data, promises to rapidly narrow the gap between sequence and function.

Antimicrobial peptides are generally encoded individually by genes that comprise homologous gene families. These genes are localized on chromosomal clusters, indicating the coevolution of different subclasses (BEVINS, 1996). To date, the defensin locus was found on chromosome 8p21-23, where the genes for both α-and β-defensins are encoded by adjacent genes. These are two peptide families with different disulfide topology but sharing a common ancestry (LIU *et al.*, 1997).

Human β-defensins described so far (DEFB1 to DEFB6) have been localized on chromosome 8.

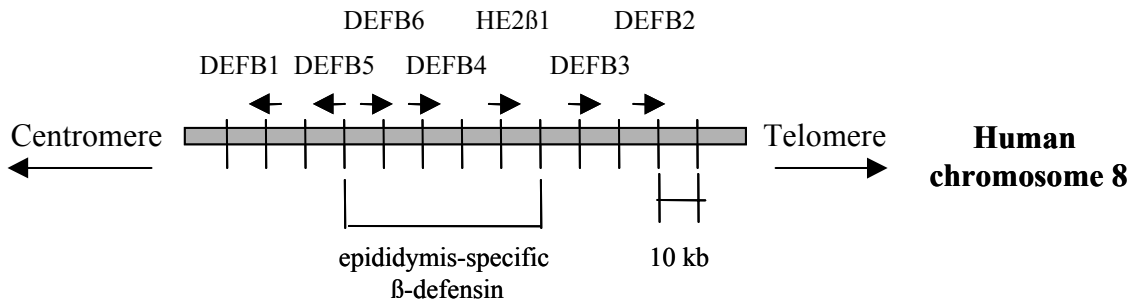


Figure 6. Schematic view of the DEFB gene cluster on chromosome 8p23.
The arrows indicate the direction of transcription of the indicated β-defensins. The epididymis-specific genes were located in the adjacent regions (YAMAGUCHI *et al.*, 2002).

Genes encoding the leukocyte α-defensins (HNP1 to HNP4) are composed of three exons. The remaining α-defensins (HD5 and HD6) have two exons. Human β-defensins DEFB1, DEFB2, DEFB3, DEFB4 and DEFB6 have a genomic structure formed by two exons. However, DEFB5 is coded by three exons, as was described for leukocyte α-defensins.

Examination of defensin mRNA has revealed that defensins are synthesized as precursor molecules, comprising a signal peptide sequence, followed by the predicted mature peptide (HARDER *et al*., 2001; GARCIA *et al*., 2001-A,-B; RODRIGUEZ-JIMENEZ *et al*., 2003). After removal of the signal peptide sequence, the predicted mature peptide is yielded. Each defensin family demonstrates substantial conservation of both amino acid and mRNA sequences in the region encoding the signalP sequence, whereas in the mRNA this sequence identity extends into the 5´-untranslated segment, which contains a region with more than 90% identity among defensins of unrelated species (SCOTT and HANCOCK, 2000).

The expression of defensin genes is necessary for the natural synthesis of peptides when the organism requires. The correlative increase of defensin mRNA with bacterial stimulation (STOLZENBERG *et al*., 1997) or inflammation (GARCIA *et al*., 2001-B) denotes their important role in the organism. The observation that relative levels of defensin proteins differ within the same or different tissues and that some are inducible while others are constitutive, suggests that defensin gene regulation is important for the maintenance of a balanced spectrum of antimicrobial activity (KAISER and DIAMOND, 2000). Therefore, the identification of the regulatory elements involved in defensin gene expression is of interest.

The expression of human α-defensins was considered as constitutive (VAN WETERING *et al*., 1999). In the case of β-defensins, the expression of DEFB1 has been considered constitutive in the kidney, pancreas, the female reproductive tract, and the airways (ZHAO *et al*., 1996; MCCRAY and BENTLEY, 1997). Other authors determined that due to the low expression of DEFB1 in human endometrial epithelial cells, a final conclusion of its regulation was prevented (KING *et al*., 2002). On the other hand, it has recently been demonstrated that in human peripheral blood, DEFB1 and DEFB2 genes were transcribed and translated following the induction of LPS or heat-inactivated bacterial cells, whereas the DEFB3 gene was not expressed. The inducible expression of DEFB1 and DEFB2 genes showed interindividual variability, but in general DEFB1 showed a higher level of expression after the mentioned stimulation. This report showed that HNP1, HNP2, and HNP3 genes were constitutively transcribed in blood cells (FANG *et al*., 2003). The human peptide hBD1 was originally isolated from hemofiltrate (BENSCH *et al*., 1995). DEFB2 is expressed in epithelia, for instance in skin and lung (HARDER *et al*., 1997) and can be induced upon epithelial stimulation by inflammatory cytokines (BECKER *et al*., 2000;

KING *et al*., 2002). In addition, bacteria and fungi also strongly enhance DEFB2 expression (HARDER *et al*., 1999). Moreover, hBD2 (HARDER *et al*., 1997) and hBD3 (HARDER *et al*., 2001) were purified from skin. DEFB3 expression was found in epithelial (e.g. lung, trachea) and non-epithelial tissues (e.g. heart, skeletal muscle) (HARDER *et al*., 2001; GARCIA *et al*., 2001-A; JIA *et al*., 2001). DEFB3 is induced by TNF-α and bacteria (HARDER *et al*., 2001), as well as γ-interferon (GARCIA *et al*., 2001-A). Recent results showed that the expression for DEFB4 was mainly expressed in testis and neutrophils, being inducible in lung epithelial cells by adding PMA (GARCIA *et al*., 2001-B). In addition, an inhibition of the stimulated expression of mRNA for hBD3 but not hBD1 and hBD2 by corticosteroids, was observed (DUITS *et al*., 2001). It is well known that corticosteroids have an important influence on the regulation of the reproductive organ function.

The group of β-defensin-like peptides was described as a human sperm antigen which was initially identified during a screening for epididymis-specific transcripts (OSTERHOFF *et al.*, 1994). These gene products are included in the mRNA species that are specifically transcribed within the proximal region of human epididymis and in no other tissue or species examined. The Human Genome Project localized the HE2 gene on the short arm of chromosome 8, directly adjacent to the DEFB3 and DEFB4 genes. The HE2 gene gives rise to multiple differentially spliced mRNA populations that encode a group of small cationic secretory peptides. Recently, the occurrence of multiple human HE2 transcripts in the human male genital tract was confirmed, including minor mRNA variants (VON HORSTEN *et al*., 2002). The homologue sequences had previously been shown only in animal species (FRÖHLICH *et al*., 2001). Three of these splicing variants named HE2C, HE2D, and HE2E share the conserved cysteine pattern of β-defensins (VON HORSTEN *et al*., 2002). Concerning the regulation of these β-defensin-like peptide genes, HE2ß2 and HE2γ are also androgen-regulated human proteins (HAMIL *et al*., 2000). They have already been shown to be secreted to the epididymal lumen and bind specifically to the sperm surface (HAMIL *et al*., 2000).

1.5 The male reproductive tract

The male reproductive system produces the male reproductive cells, the sperm, and contains an organ that deposits the sperm within the female. The male genitals include the testis, the ductuli deferentes, the ejaculatory ducts, and the penis, the prostate and the bulbourethral glands.

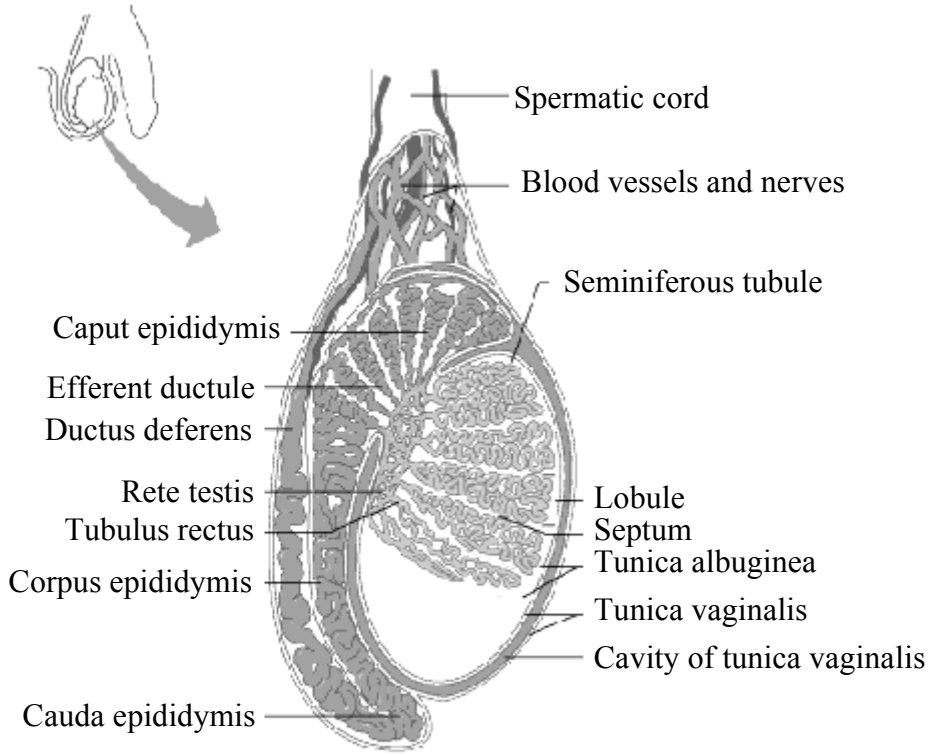


Figure 7. Vertical section of testis to show the arrangement of the different anatomical structures.

The testes are two glandular organs which secrete the semen; they are suspended in the scrotum by the spermatic cords. The testis is invested by two tunics: the tunica vaginalis and tunica albuginea (see Figure 7). The glandular structure of the testis consists of numerous lobules. They differ in size according to their position, those in the middle of the gland being larger and longer. Each lobule consists of convoluted tubes, the seminiferous tubule. They are supported by loose connective tissue which contains groups of "interstitial cells". Each tubule consists of a basement layer formed of laminated connective tissue containing numerous elastic fibers with cells between the layers and covered externally by a layer of flattened epithelioid cells. Within the basement membrane, epithelial cells are arranged in several concentric layers, which are not always clearly separated. Among these cells the spermatozoa may be seen in different stages of development (see Figure 8). The spermatogenesis process consists of three sequential phases of cell proliferation and differentiation. First, there is an extensive multiplication and proliferation of spermatogonial stem cells to produce an optimal number of spermatogonia which give rise to primary spermatocytes and to maintain a pool of stem cells. Second, the primary spermatocytes undergo the meiotic prophase, followed by the first meiotic division resulting in the formation of two secondary spermatocytes, each undergoing the second meiotic phase to produce four haploid round spermatids (ABOU-HAILA and TULSIANI, 2000). Finally, there is a gradual remodeling of the nuclear and cellular components of round spermatids during transformation into sperm cells by a process referred to as spermiogenesis (DE KRETSER and KERR, 1994). In addition to these

three layers of cells, others are seen which are termed the supporting cells (Sertoli cells). They are elongated and columnar, and project inward from the basement membrane towards the lumen of the tube. Sertoli cells contribute to provide the specific environment required by the process of spermatogenesis (ABOU-HAILA and TULSIANI, 2000).

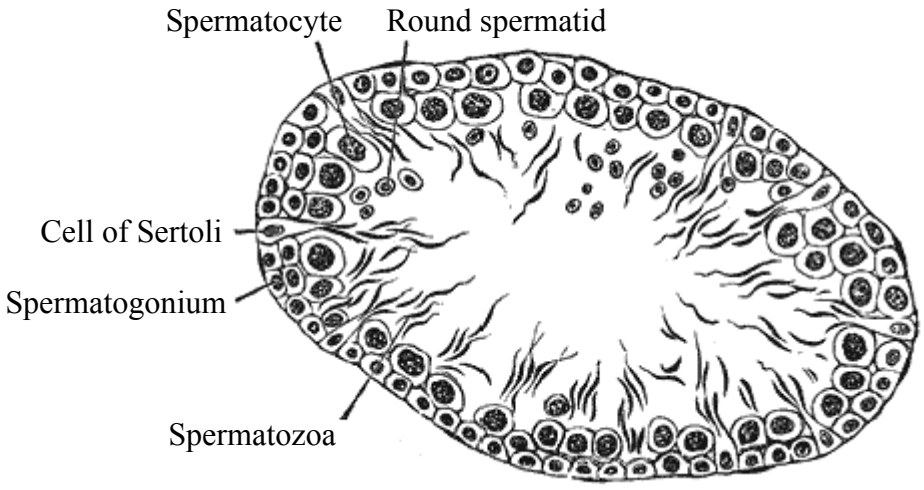


Figure 8. Transverse section of a tubule of the testis of a rat. Arrangement of the different cells of rat testis.

The epididymis is a single convoluted tubule, which is divided into three major regions based on histological appearance of the epithelium: the proximal (caput), the medial (corpus) and the distal (cauda) epididymis from which the vas deferens emerges. Spermatozoa move from the seminiferous tubules within each testis, through the rete testis and the efferent ductules, to the epididymis, and hence to the vas deferens. The epididymal duct is lined by a pseudostratified columnar epithelium, with tall columnar cells and shorter basal cells giving the appearance of two rows of nuclei. This pseudostratified epithelium lines not only the epididymis but also the vas deferens. The columnar cells are characterized by apical stereocilia. The basal cells are believed to be precursors of the columnar cells. The columnar epithelial cells lining the epididymis absorb testicular fluid, secrete a variety of factors for sperm cell maturation and maintenance, and remove damaged sperm and residual bodies (KING, 2002). The sperm migrate within the epididymal lumen (see Figure 9). In the most proximal region of the epididymis (the proximal caput), spermatozoa exhibit unidirectional orientation and are often aligned parallel to one another (DE KRETSER and KERR, 1994). This is taken as an indication that spermatozoa from this region of epididymis lack motility and their movement depends exclusively on the flow of epididymal fluid. Spermatozoa from the corpus region exhibit some degree of motility, but they are only capable of rotatory movements (ABOU-HAILA and TULSIANI, 2000). However, spermatozoa from the cauda epididymis exhibit forward motility and are considered functionally competent cells (YANAGIMACHI, 1994).

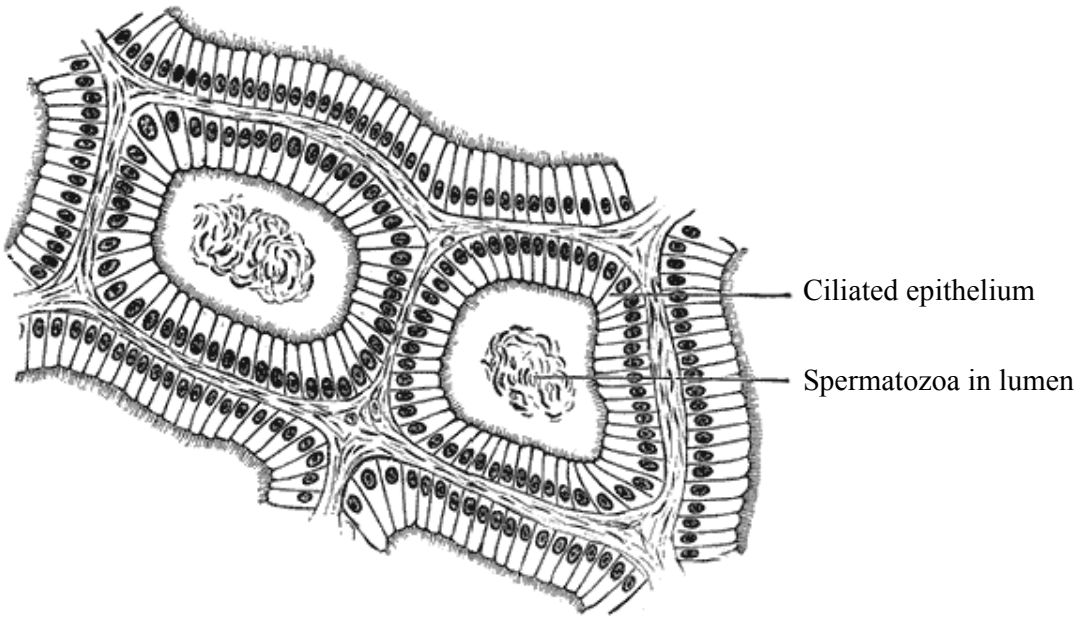


Figure 9. Section of epididymis of guinea-pig

The epididymis consists of a series of highly coiled "zones", separated by connective tissue and distinguished by epithelial cell morphology and their pattern of expression possibly reflecting the vectorial and progressive functions related to sperm concentration, maturation, transport, and storage (YEUNG *et al.*, 1993; HOLSTEIN, 1969; HAMILTON, 1990). The molecular basis of these functions is poorly understood. However, it has become increasingly clear that regional differences along the length of the duct play a role in epididymal physiology and that region-specific gene expression is involved in the formation of these differences. During the past ten years, a large number of genes have been cloned and analyzed, being involved in the establishment and maintenance of a unique and highly regionalized expression pattern along the duct. A large number of epididymis-expressed genes have been cloned and analyzed at the molecular level, most of them having been characterized by a distinct temporal and spatial expression pattern within the organ (RODRÍGUEZ *et al*., 2001). There is a long-standing interest in this regionalized pattern since it may serve as a major molecular basis underlying epididymal physiology. Most mammalian spermatozoa do not have the ability to move progressively or to fertilize an egg by the time they leave the testis. They require a posttesticular maturation process which takes place in the epididymis (BEDFORD, 1994). Most probably, factors of epididymal origin act in concert with constitutional changes to spermatozoa, which together permit full sperm function. The evidence that sperm maturation and storage require exposure to the specific microenvironment of the epididymis has been reviewed elsewere (COOPER *et al*., 1986; JONES, 1998; TURNER, 1995; HINTON *et al.,* 1996; NOLAN and HAMMERSTEDT, 1997). The functions of specific components of this microenvironment are still poorly understood.

Immunolocalization and fluorescence *in situ* hybridization have revealed that the principal cells are mainly responsible for the synthesis and secretion of proteins (HERMO *et al*., 1994). It has been suggested that may result in the sperm encountering a unique and ever-changing luminal fluid environment as they progress through the epididymis (COOPER, 1995). The order in which the spermatozoa come into contact with the specific epididymal secretions may be crucial for the resultant physiological effects (CORNWALL and HANN, 1995). Thus, the longitudinal subdivision into morphologically and functionally distinct zones may be fundamental to the process of sperm maturation and storage. The epididymal phase of sperm maturation is then followed by further changes occurring during ejaculation and finally in the female genital tract. The biochemical and functional modifications during the passage through the female tract are collectively referred to as capacitation (SUAREZ, 1987; YANAGIMACHI, 1994). Mammalian fertilization is the net result of a complex set of molecular events before penetration and fusion with the egg.

1.6 Aim of the work

Using functional genomics as a scientific strategy, a search was directed to discover new genes codifying peptides with the classical cysteine motif of β-defensins. To study their distribution and expression pattern, including the expressing cell type, gene induction and their immunolocalization in the human organism were programmed to be determined.

2. Material and methods

2.1 Material and organisms

2.1.1 Sterilization of material

Thermostable materials and solutions (glass, plastic tubes, polypropylene-tips) were sterilized for 20 min at 121°C and 1 bar in an autoclave (Bioclav 3.021001, Schütt Laboratories). Cellulose-acetate phthalate (CAP)-membrane filter, sterile filters (Filtropur, Sarstedt, 0.22 µm) were also used for solution sterilization. Metal and ceramic equipment was sterilized for 3 h at 180°C with dry heat. The solutions used in molecular biology were prepared using Milli-Q water (Milli-QUF plus, Millipore). Analysis quality water (Difco, Merck, Roth and Sigma-Aldrich) was required for several assays.

2.1.2 Instruments and devices

Precision pipettes (Eppendorf), magnetic plates and other machines were used:

Picture camera:	-EDAS 290. Documentation system with software Kodak 1D V. 3.5.0 (Kodak)
DNA-Sequencer:	-Capillary electrophoresis-sequencer PRISM 310 Genetic analyser (AppliedBiosystems [ABI])
Real-Time PCR:	-PRISM 7700 Sequence Detection System (ABI)
Microplate-Reader:	-Microplate Autoreader EL 311 (Bio-Tech Instruments)
Protein gel electrophoresis:	-Xcell SureLock Electrophoresis cell (Novex)
Spectrophotometer:	-DU 640 (Beckman)
Sterilization:	-Bioclav 3.021001 (Schütt Labortechnik)
Thermocycler:	-GeneAmp PCR System 2400 (ABI)
Ultrasonoscope:	-Vibra cell (Sonics and Materials)
Ultraturrax:	-Ultra-Turrax T25 (Janke and Kunkel)
Microscope:	-Zeiss axiophot (Zeiss)
Thermomixer:	-Eppendorf thermomixer 5436 (Eppendorf)
Hybridizer oven:	-Roller-Blot HB-3D (Techne)
Centrifuge:	-Biofuge 28 RS (Heraeus sepatech) -Hettich mikro rapid (Hettich)
Water bath:	-GFL (Labortechnik)
Speed bac:	-MC2L-60°C/Univapo 150H (Uniequip/Unicryo)
Vortex:	-IKA Labortechnik (Janke and Kunkel)
Microtome:	-HM 340 (Microm)
Refrigerator:	-UF85-360T (Colora) -Economic cooler (Bosch)
Microwave:	-700 W (Bosch)
Cell culture incubator:	-IG150 (Jouan)
Clean bench:	-8511 (Kottermann)
Miniprep culture:	-4340 illuminated refrigerator incubator shaker (Innova)
Western blotting	-XCell Sure Lock gel chamber, (Invitrogen)

2.1.3 Kits and enzymes

Different kits were used for the routine work in molecular biology, this material allows faster and safer work. Applications, commercial denominations and companies are shown (Table 2):

Table 2. Kits used in molecular biology.

Application	Kit
Isolation of genomic DNA	QIAamp DNA Mini Kit (Qiagen)
Isolation of total RNA	RNeasy Mini Kit (Qiagen)
Plasmid isolation	NucleoSpin Plus (Macherey-Nagel)
DNA purification from agarose gel	NucleoSpin Extract (Macherey-Nagel)
Purification of DNA fragments	NucleoSpin Extract (Macherey-Nagel)
DNA sequencing	PRISM BigDye Terminator Cycle Sequencing Kit (ABI)
Immunohistochemistry	Vectastain Universal Elite ABC kit (Vector Laboratories)
Real-time PCR (*TaqMan*) assay	*TaqMan* Universal PCR Master Mix (ABI) *TaqMan* PCR Core Reagent Kit (ABI)

2.1.4 Oligonucleotides

Oligonucleotides were provided by MWG-Biotech or TIB-Molbiol (Table 3).

Table 3. Overview of the different oligonucleotides.

Denomination	Oligonucleotides sequences 5´→3´	Application
β-Tub-11	TTCCCTGGCCAGCTSAANGCNGACCTNCGCAAG	Degenerated primer (sense) for housekeeping gene β-tubulin
β-Tub-21	CNCCTCGCCNGTGTACCAGTGNANGAAGGC	Degenerated primer (antisense) for housekeeping gene β-tubulin
Intro-β-Tub for	TCAGATCTTTCGGCCGGACAACTT	Primer (sense) for detecting β-Tubulin intronic sequence. Test for genomic contamination
Intro-β-Tub rev	TACCTGGATTCCAGCTTCTAGAAC	Primer (antisense) for detecting β-Tubulin intronic sequence. Test for genomic contamination
M13/pUC forward sequencing primer	AGTCACGACGTTGTAAAACGACGGCCAGT	Sequence primer for cloning vector pGEM–T
M13/pUC reverse sequencing primer	CAATTTCACACAGGAAACAGCTATGACCATG	Sequence primer for cloning vector pGEM–T
DEFB16 Race inner	CAGCACTTTTGATCATTTGGGCAGGTT	5´-Race primer for DEFB16. (Investigation of exon 1)
DEFB16 Race outer	CATATTCTCTGCAGGCGTTTCTGCACAT	5´-Race primer for DEFB16. (Investigation of exon 1)
DEFB22 Race inner	TGCTGTGGTAAATGTGGCTGGTCCTT	5´-Race primer for DEFB22. (Investigation of exon 1)
DEFB22 Race outer	TGCTGTGGTAAATGTGGCTGGTCCTT	5´-Race primer for DEFB22. (Investigation of exon 1)
DEFB23 F *in situ*	TTCATTTTGGGCTGCCTTAG	Primer (sense) for DEFB23. Confirmation of sequence and *in situ* hybridization

DEFB23 R *in situ*	ACTGGGAGCTTCATCTGCAT	Primer (antisense) for DEFB23. Confirmation of sequence and *in situ* hybridization
DEFB23 Probe TM	FAM- CTGACTCCAGGTGGCACCCAAAGATG TAMRA	*TaqMan* probe (sense) for DEFB23
DEFB23 F TM	CTTTGACTGTGCTGCTGCTCTT	*TaqMan* primer (sense) for DEFB23
DEFB23 R TM	TGTAACGGCATTTGCCATAAAG	*TaqMan* primer (antisense) for DEFB23
DEFB27 F *in situ*	GTGCAGTTCACTGGACCAAAAG	Primer (sense) for DEFB27. Confirmation of sequence and *in situ* hybridization
DEFB27 R *in situ*	TGCTCGAAGGTCATTTTAAGGAT	Primer (antisense) for DEFB27. Confirmation of sequence and *in situ* hybridization
DEFB27 Probe TM	FAM- TTGCAATTCTGCTGTTCCAGAAACCCA-TAMRA	*TaqMan* probe (sense) for DEFB27
DEFB27 F TM	CCATGGGGCTCTTCATGAT	*TaqMan* primer (sense) for DEFB27
DEFB27 R TM	CTGCAGATTTTCCTGCAATGTC	*TaqMan* primer (antisense) for DEFB27
DEFB29 F *in situ*	CAACCATGAAGCTCCTTTTTCC	Primer (sense) for DEFB29. Confirmation of sequence and *in situ* hybridization
DEFB29 R *in situ*	TGACTGATGACAGAGGAAACAGTTC	Primer (antisense) for DEFB29. Confirmation of sequence and *in situ* hybridization
DEFB29 Probe TM	FAM-CCAGGTGAACACAGAATTTATTGGCTTGAGA-TAMRA	*TaqMan* probe (sense) for DEFB29
DEFB29 F TM	TCCTATCTTTGCCAGCCTCAT	*TaqMan* primer (sense) for DEFB29
DEFB29 R TM	CATCCACATTGCAGTGATCC	*TaqMan* primer (antisense) for DEFB29
GAPDH TM for	CCTGCACCACCAACTGCTTA	*TaqMan* primer (sense) for housekeeping gene GAPDH
GAPDH TM rev	CATGAGTCCTTCCACGATACCA	*TaqMan* primer (antisense)for housekeeping gene GAPDH
GAPDH TM probe	FAM-CCTGGCCAAGGTCATCCATGACAAC-TAMRA	*TaqMan* probe (sense) for housekeeping gene GAPDH

1) S = (G + C) and N = (A + T + G + C). Nomenclature of nucleotides according to IUPAC.

2) FAM reporter (6-carboxy-fluorescein); TAMRA quencher (6-carboxy-tetramethyl-rhodamine).

2.1.5 Use of organisms and plasmids

During the present work in molecular and cell biology, the use of bacteria and plasmids was necessary, e.g. during the cloning process. The plasmid pGEM-T (Promega) allows selection by

blue/white screening. For transformation, Epicurian Coli XL10-Gold were used as competent cells.

2.2 Recombinant DNA isolation, cloning, and sequencing

2.2.1 DNA modification enzymes and restriction endonucleases

Different enzymes were used to modify DNA (during the PCR, cloning process, etc.) as well as for the reverse transcription reactions to produce cDNA from mRNA. The percentage of restriction endonucleases was less than 10% of the final volume avoiding inhibitory effects due to high concentrations of glycerin used to store the enzymes.

Table 4. Enzymes used during DNA manipulation.

Enzyme	Commercial denomination
Reverse transcriptase	SuperScript II RT (Life Technologies)
DNA polymerase	*Taq* Platinum DNA-polymerase (Life Technologies) Ampli*Taq*-Gold (ABI)
DNA nuclease	DNAse I Amp Grade (Life Technologies)
Restriction endonucleases	*Pst* I, *Not* I (Life Technologies)
RNase-inhibitor	RNasin (Promega)
DNA ligase	T4 DNA ligase (Promega)

2.2.2 Standard polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a rapid procedure for *in vitro* enzymatic amplification of specific segments of DNA (SAIKI *et al.*, 1985). PCR has spawned a multitude of experiments that were previously impossible. The present work includes different applications, for instance the amplification of fragments from genomic DNA or cDNA as well as nucleotide sequencing.

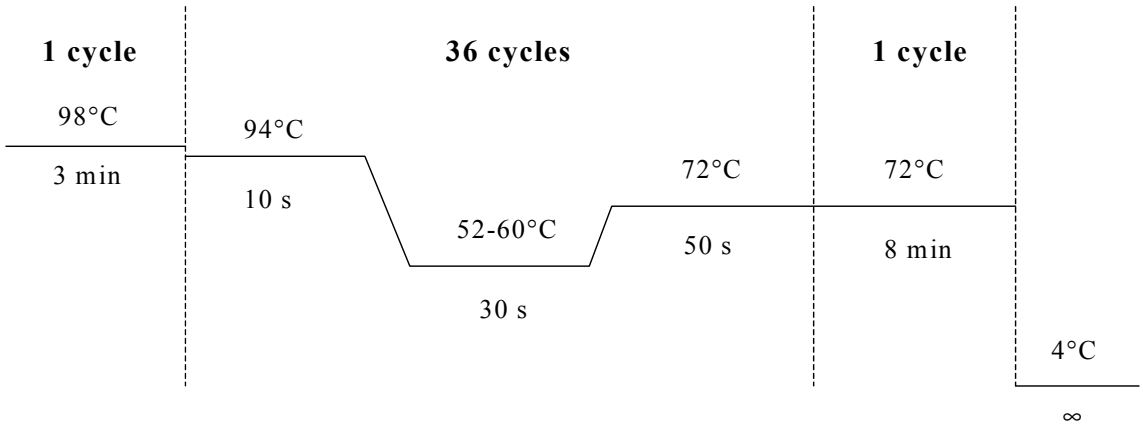
Components of the PCR reaction:

10%	10 × PCR-buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl)
2 mM	$MgCl_2$
200 nM	Oligonucleotide primer (sense and antisense)
200 mM	dNTPs (200 mM each of dATP, dCTP, dGTP and dTTP)
7.5 – 200 ng	Template DNA (cDNA, plasmid or genomic DNA)
0.75 U	*Taq* Platinum DNA-polymerase (Life Technologies)
add until 100%	ddH_2O

Final volume in a standard PCR was 50 µl**.**

The parameters of a PCR depend on the characteristics of the sequence object of study (e.g. length of oligonucleotides used, length of amplicons, etc). A standard PCR was followed according to the following guidelines:

1.	1 cycle		
	Initial denaturation of the DNA	98°C	3 min
2.	36 cycles		
	Denaturation	94°C	10 s
	Annealing of the primers	52-60°C	30 s
	Extension	72°C	50 s
3.	1 cycle		
	End of the extension	72°C	8 min
4.	End of the reaction	4°C	∞



The annealing temperature of DEFB23, DEFB27, DEFB29 for confirmation of the mRNAs was 55°C for 12 s, followed by an extension step at 72°C for 45 s. The bands of interest were amplified using cDNA produced from mRNA extracted from human testis. The polymerase chain reaction was performed using a GeneAmp PCR System 2400 Thermocycler (ABI), and *Taq* platinum DNA-polymerase. This enzyme has the advantage of thermostability, and the ability to stand the repeated heating and cooling inherent in PCR and to synthesize DNA at high temperatures with an efficiency of approximately 1000 bp/min.

2.2.3 Colony PCR

Colony PCR is a very efficient method to identify the clones containing the plasmid with the insert of interest and to differentiate them from the other clones not incorporating it. This technique constitutes an alternative method of selection to the classical way of restriction analysis. After transformation using competent cells, the positive colonies are selected according to their color. The pGEM-T cloning vector produces a functional lacZ- gene α fragment that allows the formation of a β-galactosidase enzyme which results in the formation of blue colonies on Xgal/IPTG indicator plates. Cloning of DNA fragments into the polylinker site prevents

production of a functional lacZ-α fragment (white colonies), allowing a rapid identification of positive clones. The bacteria were cultivated onto an agar plate (supplied with 100 µg/ml ampicillin) and simultaneously incorporated into the PCR tube. The amplification of the genetic material was achieved by using pUC/M13 sense and antisense oligonucleotides (Table 3). These primers include the polylinker regions of the plasmid used (pGEM-T) amplifying 250 bp (in the case of negative colonies) or 250 bp plus the length of the insert (in the case of the positive colonies).

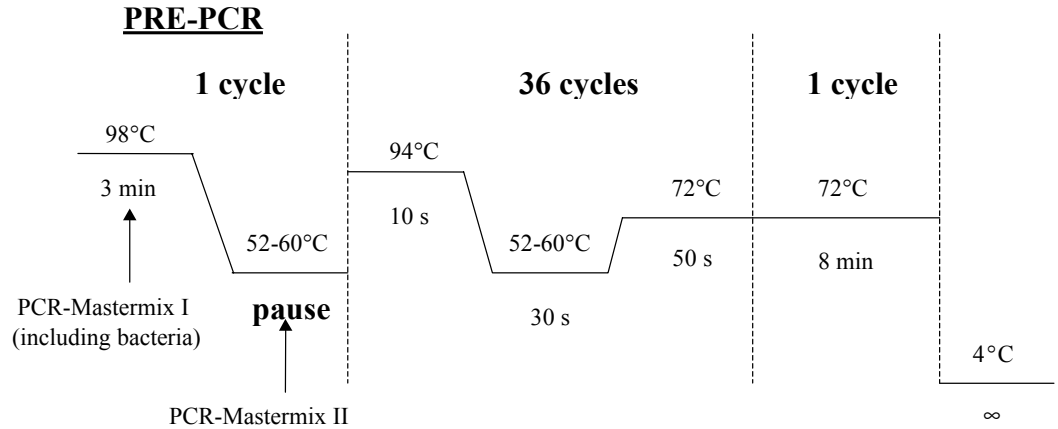
PCR premastermix I (amount per reaction):

2 µl 10 × PCR buffer
0.8 µl 50 mM $MgCl_2$
17.5 µl ddH_2O

PCR mastermix II (amount per reaction):

1 µl	10 × PCR buffer
0.6 µl	$MgCl_2$ (50 mM)
0.25 µl	dNTPs (10 mM)
0.25 µl	Oligonucleotide primer (10 µM)
7.5 µl	ddH_2O
0.15 µl	*Taq* Platinum DNA-polymerase

Using sterile material, the chosen bacterial colonies were cultivated by applying the selected clones with a tip onto the surface of an agar plate. The same tip was subsequently used to provide the template in an individual PCR tube with 20 µl PCR premastermix I. Then the cycler heated the samples at 98ºC for 3 min before decreasing the temperature to 52-60ºC. The thermocycler was paused in order to include 10 µl of the second part of the mastermix (PCR-mastermix II) during the pre-PCR. The rest of this process coincides with the standard PCR described previously (see 2.2.2) except for the initial denaturation. Once the procedure was finished, the positive clones were selected, inoculated and grown in 3 ml LB medium with ampicillin at 37°C/ overnight.

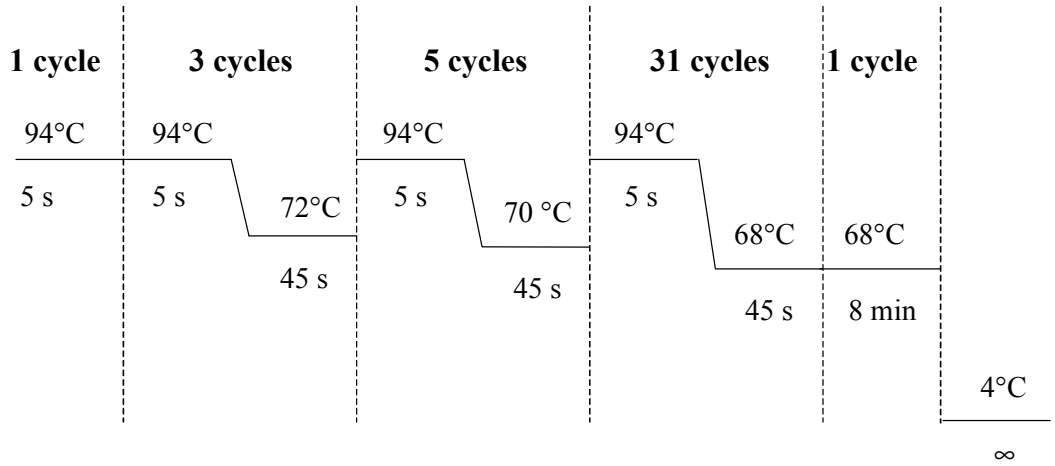


2.2.4 RACE-PCR

Rapid amplification of 5´- cDNA ends by polymerase chain reaction (5´- RACE-PCR) was used to determine unknown 5´- cDNA ends. A RACE PCR kit (Clontech) was used to identify exon 1 for pseudogenes DEFB16 and DEFB22, and to complete their nucleotide sequences in the 5'-direction (GLADMAN *et al.*, 1988). Thus, cDNA amplification was performed with a specifically designed antisense primer from the known sequence of public DNA clones (ESTs). The use of *Taq*-polymerase has the effect of adding a poly A to the 5´-end of amplicons, that would facilitate the assembly of a complementary anchor (dT) 16 V primer (AP1) in this position of the sequences. This fact creates a double strand and, in consequence, an amplification of the unknown 5'-termini. The specificity of this reaction was increased by a second PCR (inner-PCR), using the specific DEFB16, DEFB22 race primer and the inner AP2 primer. AP2 is complementary to AP1, assembling in the inner location. Both reactions were executed in accordance with the manufacturers´ instructions. The temperature of annealing during the extension decreased progressively by two degrees in all subsequent stages, accepting the possibility of non-specific amplifications. On the other hand, this PCR program guaranteed obtaining amplification products.

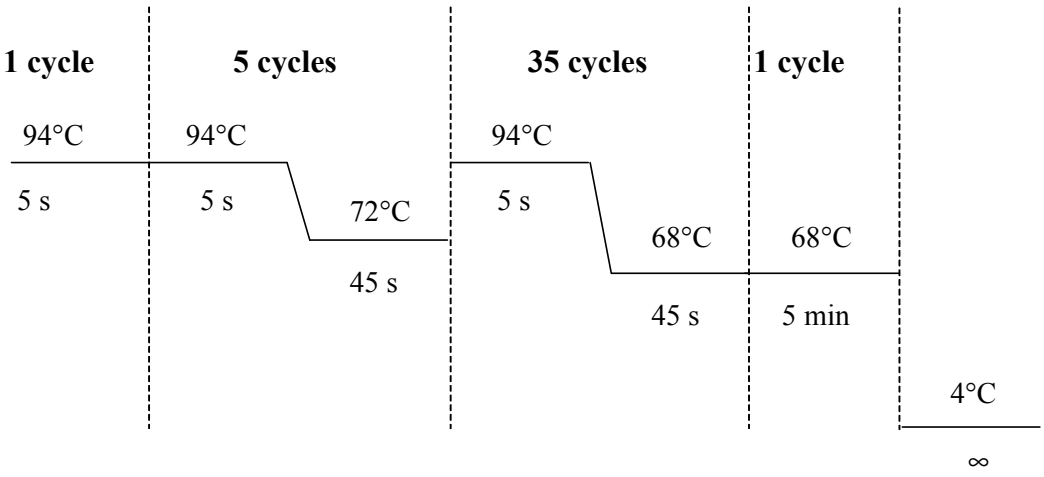
Outer PCR

1.	Initial denaturation of the mix	94°C	5 s
2.	3 cycles		
	Denaturation	94°C	5 s
	Annealing of the primers and extension	72°C	45 s
3.	5 cycles		
	Denaturation	94°C	5 s
	Annealing of the primers and extension	70°C	45 s
4.	31 cycles		
	Denaturation	94°C	5 s
	Annealing of the primers and extension	68°C	45 s
5.	1 cycle		
	Annealing of the primers and extension	68°C	8 min
6.	End of the reaction	4°C	∞



Inner PCR

1.	Initial denaturation of the mix	94°C	5 s
2.	5 cycles		
	Denaturation	94°C	5 s
	Annealing of the primers and extension	72°C	45 s
3.	35 cycles		
	Denaturation	94°C	5 s
	Annealing of the primers and extension	68°C	45 s
4.	1 cycle		
	Annealing of the primers and extension	68°C	5 min
5.	End of the reaction	4°C	∞



2.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis is a simple and highly effective method for separating, identifying, and purifying DNA fragments. A gel is prepared with an appropriate concentration of agarose for the size of DNA fragments to be separated. Electrophoresis was performed at 80-120 V, for a period of time that allowed optimal separation. For visualization of DNA, ethidium bromide (0.1 µg/ml) was applied previously into the gel. The fragments can be visualized directly upon UV light and photographed with a Kodak EDAS 290 documentation system. DNA ladders (1 kbp or 100 bp ladder, Life Technologies) were used for estimation of fragment length.

Ethidium bromide solution: 1 mg/ml Ethidium bromide in ddH_2O.

50 × TAE buffer:

2 M	Tris
100 mM	EDTA
1 M	Glacial acetic acid

5 × Loading buffer

40%	Glycerin
20 mM	Tris-HCl [pH 8.0]
2 mM	EDTA
0.1% (w/v)	Bromphenol blue

2.2.6 Extraction of DNA fragments from agarose

DNA extraction was performed with the aid of NucleoSpin Extract Kits from Macherey-Nagel (VOGELSTEIN and GILLESPIE, 1979). Using a clean scalpel, DNA fragments were excised from the gel. For each 100 mg agarose gel, 300 µl buffer NT1 were added in order to dissolve the agarose. The samples were incubated for 10-15 min at 50°C, loaded on a NucleoSpin Extract tube and placed in a 2 ml centrifuge tube. After a centrifugation step at 6000 × g for 60 s, the flowthrough was discarded. The NucleoSpin Extract tubes were again placed in the centrifuge tube. Then, 700 µl buffer NT3 were added and the samples were centrifuged at 12.000 × g for 60 s to remove buffer NT3. NucleoSpin Extract tubes were again placed in the centrifuge tube and centrifuged at 12.000 × g for 2-5 min, to remove of residual ethanol which would inhibit subsequent reactions. After placing the NucleoSpin Extract tubes in a clean 1.5 ml centrifuge tube, 50 µl of ddH_2O were added to elute the DNA by centrifuging at 12.000 × g for 60 s.

2.2.7 DNA ligation

In general terms, cloning vectors are selected according to the size of the insert of interest. During this work, the pGEM-T vector (Promega) was used routinely. The PCR fragments were modified with *Taq* platinum DNA polymerase (Life Technologies). This enzyme adds a nontemplated nucleotide (usually dA) to the ends of PCR fragments. The vector pGEM-T (Promega) provides terminal thymidines that fit with the nontemplated nucleotide dA allowing the ligation process.

Composition of the ligation reaction:

6 µl (0.1 to 5 µg)	Extracted products from agarose gels
1 µl	10 × Ligation buffer
2 µl (20 ng)	pGEM-T (Promega)
1 µl (3 U)	T4 DNA ligase (Promega)

After mixing all reagents properly, ligation was performed overnight at 4°C. Then, the vector (insert included) was transformed into Epicurian Coli XL10-Gold Ultracompetent Cells.

2.2.8 Competent cell preparation

The production of transformational competent cells was performed by incubation of bacteria with $MgSO_4$ (NISHIMURA *et al.*, 1990). The solution was sterile-filtered through an 0.2 µm filter before adding it to the LB-Medium (Medium A). This solution was prepared freshly. Then, 1 ml of medium A was removed and stored at –20°C while the remaining medium was stored at room temperature, overnight.

Simultaneously, an overnight culture of Epicurian Coli XL10-Gold Ultracompetent Cells in 5 ml of LB-Medium (without antibiotics) was incubated at 37°C with shaking at 200 rpm.

The next day, 1 ml of the culture obtained overnight was incubated in 100 ml medium A, at 37°C. The cells grew steadily until they reached $OD_{578nm} = 0.55$ (blank: LB-medium). Afterwards the culture was placed on ice for 10 min and was subsequently centrifuged at 4000 × g, at 4°C in 50 ml tubes. Then the supernatant was discarded and the pellet was resuspended carefully in 1 ml medium A previously stored at –20°C. After complete resuspension of the pellet, 5 ml of medium B was added, drop by drop and under constant agitation of the tube. Afterwards, the preparation was placed on ice again and aliquoted in 100 µl portions into prechilled 1.5 ml tubes. The aliquots were deposited as deeply as possible at –80°C. The prepared *E.coli* cells became competent after being kept at –80°C for approximately one week.

LB (Luria-Bertani) medium (see cell and yeast culture).

Medium A:

LB medium with:	10 mM $MgSO_4$
	0.2% Glucose

$MgSO_4$-solution and glucose were filtered previously.

Medium B:

LB medium with:	36% Glycerin
	12% Polyethyleneglycol (PEG 8000)
	12 mM $MgCl_2$

The PEG 8000 was dissolved in glycerin and added to the LB-Medium (with magnesium).

2.2.9 Bacterial cell transformation

Aliquots of 100 µl competent cells were slowly defrosted and 5 µl from the ligation reaction were included in the tube containing the bacteria. The solution was subsequently incubated on ice for 30 min, to allow the recombinant plasmid DNA to attach to the bacterial membrane. After

warming the samples to 42°C for 90 s, the tubes were immediately transferred on ice for 5 min. The goal of this step is to produce a thermo-shock, allowing the recombinant plasmid to be included into the cells. Subsequently, 900 µl of SOC-Medium was added to the ligation reaction tube for further incubation at 37°C for 60 min, with gentle shaking. The bacteria were centrifuged at 400 × g for 5 min. The supernatant was removed and 40 µl of the remaining solution were applied on an agarose plate, containing 100 µg/ml ampicillin together with 40 µl of IPTG and 40 µl of X-Gal. Ampicillin selects the growing colonies with resistance to this antibiotic. The three compounds were mixed and spread on the plates. The selected colonies were used to perform colony PCR (see 2.2.3).

SOC Medium:

2%	Casein
1%	Yeast extract
2.5 mM	KCl
10 mM	$MgCl_2$
10 mM	$MgSO_4$
10 mM	NaCl
20 mM	Glucose

The medium was autoclaved; pH 7.0 was adjusted by adding 5 N NaOH.

IPTG solution (sterilized by filtration):

100 mM isopropyl-1-thio-β-D-galactoside in ddH_2O

X-Gal solution:

2% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactoside in N. N-dimethylformamide

Selection-agar plate:

LB-medium with:	1.5% agar (Sigma A-5054)
	100 µg/ml ampicillin

Afterwards the solution was autoclaved, 100 µg/ml ampicillin was added when the temperature was below 50°C and pH 7.4 was adjusted using 1 N NaOH.

2.2.10 Miniprep double-stranded DNA isolation

The isolation of plasmid DNA from *E. coli* cells, containing recombinant plasmids, was performed using the alkaline lysis procedure (BIRNBOIM and DOLY, 1979). Bacteria were cultured overnight in 3 ml LB-medium with 3 µl ampicillin (100 µg/ml). Subsequently, 2 ml of culture were centrifuged at 12.000 × g for 30 s. Lysis was performed by treatment with a solution containing sodium dodecyl sulfate (SDS) and NaOH, (SDS denatures bacterial proteins, NaOH denatures chromosomal DNA). NucleoSpin Plasmid, (Macherey-Nagel, Germany) is designed for the rapid small-scale preparation

of high-purity plasmid DNA. After centrifugation, the pellets were resuspended carefully in 250 µl buffer A1 including RNase A. The lysis buffer A2 was applied to the preparations for 5 min at room temperature. The lysis was stopped by adding buffer A3. The suspensions were always mixed gently by inverting the tubes 6-8 times and incubated on ice for 5 min. The resulting solutions were centrifuged at 12.000 × g, for 10 min, at 4°C. Thereby, bacterial proteins and genomic DNA were sedimented. Placing NucleoSpin columns in a 2 ml centrifuge tube, the supernatants from the previous step were loaded onto the columns and centrifuged at 12.000 × g for 60 s. Then, the flowthroughs were discarded. The columns were washed with 0.5 ml buffer AW. Columns were centrifuged at 12.000 × g for 60 s and washed by adding 700 µl of buffer A4. After centrifugation at 12.000 × g for 3 min, the flowthroughs were removed. This step was repeated in order to eliminate residual ethanol. Placing the NucleoSpin columns in a new centrifuge tube, the DNA was eluted by adding 50 µl water and a final centrifugation was performed at 12.000 × g for 1 min.

2.2.11 Sequencing of DNA

The dideoxy or enzymatic method (SANGER *et al*., 1977) was used to identify DNA fragments. The sequencing reaction was performed using a GeneAmp PCR System 2400 Thermocycler. A synthetic oligonucleotide primer was annealed on a single-strand DNA template after a previous denaturation step. The sequencing reactions were always produced using a PRISM Big Dye Terminator Cycle Sequencing Kit (ABI).

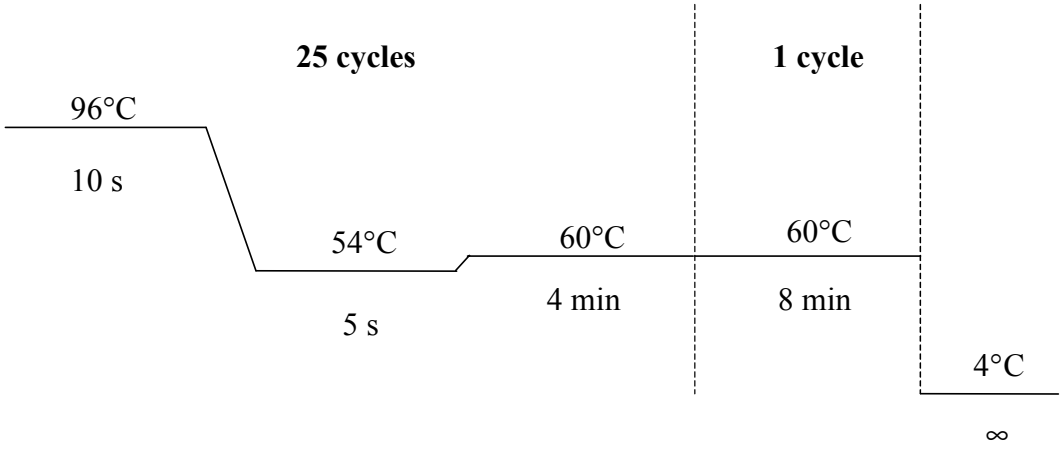
Components of a sequencing reaction:

4 µl BigDye Terminator Ready Reaction Mix
1 µl Oligonucleotide primer (10 pmol/µl)
x µl template DNA (7.5 ng)

Add ddH2O until 20 µl.

Program of temperatures used for amplification of the sequences.

1.	25 cycles		
	Denaturation	96°C	10 s
	Annealing of the primers	54°C	5 s
	Extension	60°C	4 min
2.	1 cycle		
	End of the extension	60°C	8 min
3.	End of the reaction	4°C	∞



Once the sequencing reaction was finished, 80 µl ddH_2O were added to every single reaction. The precipitation of the DNA was performed at 4°C by adding the mix to a 1.5 ml tube containing 15 µl 2 M sodium acetate solution (pH 5.2) and 250 µl 95% ethanol. Tubes were incubated on ice for 10 min. The samples were centrifuged at 12000 × g for 15 min to allow sedimentation of DNA. After discarding the supernatant, pellets were washed with 250 µl 70% ethanol and centrifuged again at 2000 × g for 5 min. The resulting products were freeze-dried and resuspended again in 20 µl ddH_2O. At this point the samples were applied to the PRISM 310 Genetic Analyzer (ABI) sequencer. The parameters for the electrophoresis were 30 s of probe injection and 15 kV at 50°C. The sequences were analyzed using V3.4.1-Software (ABI).

2.3 Analysis of gene expression

RNA is an unstable molecule and the major source of failure in any attempt to produce RNA is contamination by ribonucleases. RNases are very stable enzymes and generally require no cofactors to function. Therefore, a small amount of these enzymes in RNA preparation would create future problems. To avoid tedious and current time-consuming methods involving the use of toxic substances, RNeasy Kit (Qiagen) was used, providing a fast and simple method for obtaining RNA.

2.3.1 Isolation of total RNA from animal tissues and cells

For the isolation of RNA from animal tissues the following tissues were purchased from Clontech Laboratories (Palo Alto, CA. USA): small intestine, skin, testis (including epididymis), placenta, trachea, heart, skeletal muscle, liver, thyroid gland, pancreas, uterus, lung, kidney, salivary gland, prostate, brain, cerebellum, adrenal gland, and mammary gland. Tissue samples from esophagus, jejunum, colon, rectum, bladder as well as from different stomach segments (antrum, fundus, corpus) were kindly provided by Professor Dr. Markus Buechler (Heidelberg, Germany) and PD Dr. Helmut Friess (Bern, Switzerland). For every organ object of study, 500 mg were homogenized. For this purpose, tissues were placed in a mortar with liquid nitrogen and divided into

small pieces. Tissues were shredded using an Ultra-Turrax T25 rotor-stator homogenizer. RNA isolation from cell lines was performed using 500 µl lysis buffer, (RLT-buffer, Qiagen) and 1% β-mercaptoethanol. Prior to isolation, QIAshredder columns (Qiagen) were used for material homogenization. Culture cells or tissues were homogenized in a denaturing solution containing 4 M guanidine thiocyanate and β-mercaptoethanol in the lysis buffer, causing RNAses and protein denaturation. One volume of 70% ethanol was then added to the resulting mixture. Subsequently, 700 µl of sample were applied to an RNeasy mini spin column placed in a 2 ml collection tube and centrifuged at $8000 \times g$ for 15 s. To remove undesirable compounds, 700 µl buffer RW1 were added onto the RNeasy column and centrifuged at $8000 \times g$ for 15 s. An ethanolic solution (buffer RPE) was added in order to achieve a clean extraction of RNA. The resulting mixture was centrifuged at $8000 \times g$ for 15 s. In addition, and after removing the flowthrough, the samples were centrifuged at $8000 \times g$ for 2 min for the removal of residual ethanol which would interfere with subsequent reactions. Afterwards, the RNeasy columns were transferred to new 1.5 ml collection tubes. RNase-free water was added and RNA was eluted by centrifugation at $8000 \times g$ for 60 s. Finally an aliquot of this RNA solution was used for measuring the concentration by photometric analysis (see 2.3.2). The resulting RNA solution was stored at -80°C.

2.3.2 Determination of DNA/RNA concentration

For some experiments, e.g. reverse transcription and DNA sequencing, it is necessary to determine the nucleic acid concentration in solution. For this purpose, the absorption of an aqueous solution was measured in a quartz tray, with 1 cm of thickness at a wavelength of 260 nm. The nucleic acid concentration (C) was determined by the following formula:

$$c[ng/\mu l] = OD_{260} \times \varepsilon \times V$$

The extinction-coefficient ε for single-strand DNA or RNA is 0.04 and for double-strand 2dDNA is 0.05, while V describes the dilution. OD_{260} is the optical density at 260 nm.

In addition, the purity of a nucleic acid preparation can be determined by UV absorption. The ratio from the measured optical densities at 260 nm and 280 nm (OD_{260}/OD_{280}) indicates whether the nucleic acid solution is contaminated by protein remain. Pure DNA preparations indicate a ratio of 1.8, whereas clean RNA solutions would reflect a ratio of 2.0.

2.3.3 Synthesis of cDNA from RNA

The sensitive method of the PCR can be applied not only to genomic DNA, but also to cDNA molecules to indirectly examine RNA transcripts in different tissues. A prerequisite for such an investigation is transcribing RNA into complementary DNA (cDNA) using a viral reverse transcriptase. To avoid contamination with genomic DNA, RNA preparations were routinely digested with DNase I before use in cDNA synthesis. Then, 1.5 µg freeze-dried RNA were solved in 7 µl RNase-free water. Subsequently, a mix formed by 1 µl RNasin (Promega), 10 × DNase reaction buffer and 5 µl DNase I (Life Technologies) was added. DNA digestion was stopped after 15 min by adding 1 µl 25 mM EDTA. After incubation at 65°C for 10 min the enzymes involved were completely inactivated. The solutions were treated with 500 ng random hexamer primers. The mix was denatured at 70°C for 10 min and rapidly cooled down on ice for 5 min, in order to enable assembly of the hexamer primers. Subsequently, 6 µl of 5 × 1 st strand buffer, 3 µl of 0.1 M dithiothreitol (DTT) and 1 µl dNTP mix (10 mM) were added and mixed carefully. The solution was incubated for 2 min at 42°C. The reverse transcription started by adding 200 U SuperScript II (Life Technologies). The resulting products were incubated for 50 min at 42°C. The reaction was stopped by inactivating the enzyme at 70°C for 15 min. The concentration of the complementary DNA produced was calculated from the RNA amount used.
An estimation of the cDNA quality was determined in two following control experiments. A PCR with a β-tubulin pair of primers checked the efficiency of cDNA, and a second PCR with intronic β-tubulin-primers tested putative genomic contamination.

2.3.4 Real-Time PCR (*TaqMan*)

For the accurate and reproducible quantification of transcripts in cells and tissues, real-time PCR (*TaqMan*) was performed using a PRISM 7700 Sequence Detection System (ABI). The principle of this method is based on the use of the 5´nuclease assay described by HOLLAND *et al*., 1991. It consists of detecting specific PCR products based on the use of dual-labeled fluorogenic probes (LEE *et al*., 1993). The probe is designed to hybridize within the target sequence. Then a signal is generated and accumulated during PCR cycling in a manner proportional to the concentration of amplification products. The method uses the 5´nuclease activity of *Taq* polymerase to cleave a nonextendible hybridization probe during the extension phase of PCR. The measurement of fluorescence in each sample provides a homogeneous signal, which is specifically associated with the amplified target and quantitatively related to the amount of PCR products. High specificity and sensitivity in nucleic acid quantification was achieved by choosing adequate primers and probe sequences. Annealing temperature of the chosen oligonucleotide primers was around ten degrees

below the annealing temperature of the probe. Oligonucleotides must not contain many Cs and Gs at the 5´or 3´ends of the primers and probes, because it would impair the correct assembly of the sequences. These rules are important for avoiding problems such as inefficient cleavage of probes.

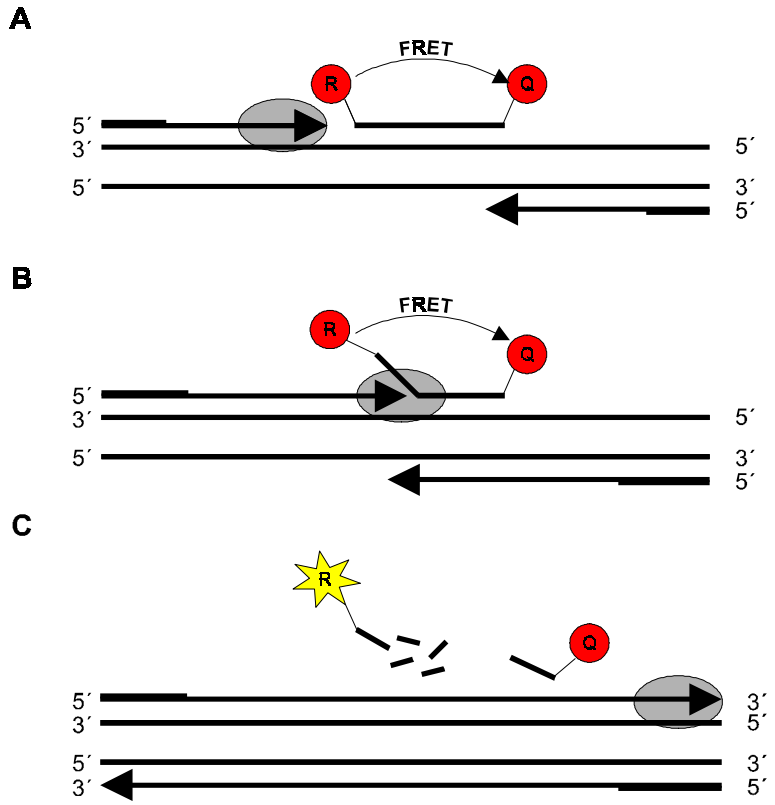


Figure 10. Principle of the *TaqMan* PCR.
(A) At the beginning of the PCR the oligonucleotide probe labeled with reporter dye (R) and quencher (Q) hybridize to the target sequence. By the spatial proximity of the two fluorescence dyes a fluorescence resonance energy transfer (FRET) takes place, and therefore the fluorescence signal is suppressed. (B) During the elongation phase the *Taq* DNA polymerase meets the bound probe. A transition structure during the displacement reaction activates the 5'- 3'- exonuclease activity of the *Taq* DNA polymerase and leads to the hydrolysis of the probe (C). The distance between reporter coloring material and quencher, increased now, allows the FRET to be interrupted and a fluorescence signal becomes measurable. The *Taq* DNA polymerase is symbolized by a grey ellipse, assembled oligonucleotide primer by thick lines. Energy of the reporter dye material becomes measurable as a fluorescence signal (λ=518 nm).

The dual-labeled fluorogenic probe is formed by a fluorescent dye which serves as a reporter FAM (6-carboxy-fluorescein) and its emission spectrum is quenched by the second fluorescent dye, TAMRA (6-carboxy-tetramethylrhodamine). When the probe is intact, fluorescent energy transfer occurs and the reporter dye fluorescent emission is absorbed by the quenching dye. During the extension phase of the PCR cycle, the fluorescent hybridization probe is cleaved by the 5´-3´ nucleolytic activity of the DNA polymerase. On cleavage of the probe, the reporter dye emission is no longer efficiently transferred to the quenching dye, resulting in an increase of the reporter dye fluorescent emission spectra.

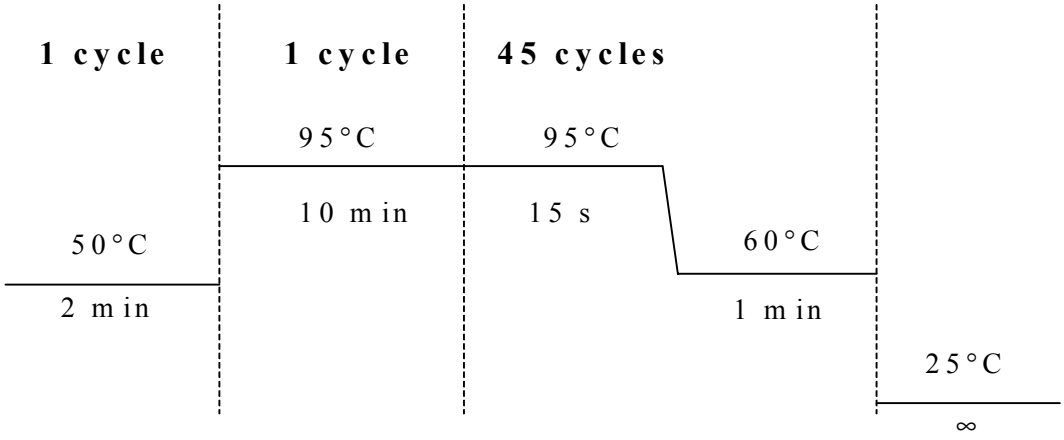
The nuclease degradation of the hybridization probe releases the quenching of the FAM fluorescent emission which is measured by the detection system. To calculate the quantity of output cDNA, the amplification plot is examined early in the reaction, at a point that represents the exponential phase of product accumulation, in which the fluorescence intensities are detected according to their wavelengths. This is achieved by assigning an arbitrary threshold that is based on the variability of the base-line data. Once the threshold is chosen, the point at which the amplification plot crosses the threshold is defined as CT (threshold cycle). CT is reported as the cycle number at this point. The CT value is predictive of the quantity of input target. Because of the necessity of three different oligonucleotides (2 primers and the probe), the dependency of a detectable fluorescence signal makes the real-time PCR a highly specific assay. Moreover, a possible amplification of genomic sequence was additionally prevented by the use of probes which hybridize at the exon-intron boundary position.

The modifications of the fluorescence intensities during a real-time PCR were measured with the help of the PRISM 7700 Sequence Detection of System (ABI). All necessary probes were labeled with 6-carboxy-fluorescein (FAM) as reporter and with 6-carboxy-tetramethyl-rhodamine (TAMRA) as quencher, becoming excited by the ionized argon laser of the PRISM 7700 (λ=488 nm). The emission spectra of the fluorescence coloring materials were measured automatically within the range of 500-660 nm and converted by a CCD camera (charge-coupled device) into data points, being interpreted by computer.

Oligonucleotide primer and probes were derived with the program primer express (ABI) and from the companies MWG (primer) and TIB MOLBIOL (probes). The use of an endogenous reference gene can also prevent errors which result from an inaccurate estimation of total RNA concentration and quality in the initial sample. The expression of a housekeeping gene, in this case GAPDH (glyceraldehyde-3-phosphate-dehydrogenase), is commonly used as an endogenous reference mRNA target.

TaqMan PCR program:

1.	1 cycle Uracil-N-Glycosylase-reaction	50°C	2 min
2.	1 cycle Initial denaturation	95°C	10 min
3.	45 cycles Denaturation	95°C	15 s
	Annealing process and Amplification of sequences	60°C	1 min
4.	End of the reaction	25°C	∞



The absorption spectra were obtained during the amplification of the target sequence.

For each PCR reaction the following reagents were needed:

12.5 µl 2 × *TaqMan* universal PCR master mix (ABI)
250 nM Oligonucleotide primer (250 nM each primer)
250 nM Probe (5´-FAM/3´-TAMRA labeled oligonucleotide)
2.0 µl cDNA (Template)

Final volume: 25 µl.

2.3.5 Quantification of endogenous expression

The quantification of the transcription of DEFB23, DEFB27 and DEFB29 was achieved by using a standard curve as the basic method of quantification. The external calibration curve requires that the absolute concentration of the standard is known by some independent method (photometric analysis).

Recombinant DNA plasmids, containing the respective amplicon of interest, were used to prepare absolute standards. Thus, serial dilutions of recombinant plasmid containing a copy number of the target sequence (DEFB23, DEFB27, DEFB29 or housekeeping gene) were prepared. The correlation of the dilutions in the standard curve is indicated by a correlation coefficient which must be R > 0.97 to provide an exact evaluation method. The number of copies/µl of the molecule of interest can be determined by the following mathematical formula.

I. Transformation of the concentration [µg/µl] in [pmol/µl]:

$$\mu\text{g DNA} \times \frac{pmol}{660\ pg} \times \frac{10^6\ pg}{1\ \mu g} \times \frac{1}{N} = pmol\ \text{DNA}$$

II. Transformation of [pmol/µl] in [number of molecules /µl]:

$$[\text{pmol DNA}/\mu l] \times N_A = [\text{molecules of target sequence}/\mu l]$$

N = Number of nucleotide pairs, including insert; $N_A = 6{,}023 \times 10^{23}$ (Avogadro´s number) (number of copies /mol); 660 mol/g is the average molecular weight of a nucleotide pair.

2.4 Culture of human cells and microorganisms

2.4.1 Culture of prokaryotic cells and yeast

The cultivation of bacteria was based on the general guidelines indicated by SÜßMUTH *et al*., 1999. The description of the germs used and their growth conditions are shown in Table 5.

Table 5. Germs used for the investigation of the antimicrobial activity of hBD23, hBD27, and hBD29.

Bacteria	Strain	Medium	Gram staining	Temperature of culture
Staphylococcus carnosus	TM300	LB	+	37°C
Staphylococcus aureus	ATCC25923	TSB	+	37°C
Streptococcus pneumoniae	DSM11865	TSB	+	37°C
Escherichia coli	DSM96440	LB	-	37°C
Escherichia coli	DSM1103	LB	-	37°C
Klebsiella pneumoniae	DSM681	LB	-	37°C
Pseudomonas aeruginosa	DSM1128	LB	-	37°C

Culture media:

LB (Luria Bertani):

1.0% Casein (Sigma C-0626)
0.5% Yeast extract (Sigma Y-4000)
0.5% NaCl

Solids were disolved in water (pH 7.4 adjusted by adding 1N NaOH). The solution was autoclaved.

TSB-Medium (Tryptic Soy Broth):

0.3% Tryptic Soy Broth (Sigma T-8261)
99.7% Bidest water.

The solid was disolved (pH 7.2 adjusted by adding 5 N NaOH). The solution was autoclaved.

2.4.2 Cell culture

2.4.2.1 Cell culture conditions

Human cell lines were cultured following the recommendations summarized by LINDL *et al*., 2000. The cells were cultured under standard conditions at 37°C and 5% CO_2. Cultures stored in liquid nitrogen were thawed at 37°C in a water bath and washed with culture medium (20% FCS) in order to remove the freezing medium (DMSO).

After sedimentation for 5 min at $120 \times g$, cells were resuspended in 20 ml culture medium and transferred to 75 cm² culture flasks. When 80-90% confluence was reached, cells were washed twice with PBS and treated with 3 ml trypsin/EDTA solution for 2-4 min at 37°C.

The time of tryptic treatment depends on the cellular type. The reaction was stopped by adding 10 ml medium containing 10% FCS. The supernatant was discarded after centrifugation. Then, cells were resuspended in fresh culture medium. Surplus cells were frozen, stored at -80°C for 48 h and transferred to liquid nitrogen (-196°C) in suitable freezing containers (Cryovial, Roth).

Freezing medium: The appropriate cell line medium plus:

20% FCS
2 mM L-Glutamine
100 µg/ml Penicillin/Streptomycin
10% DMSO

The addition of 10% of DMSO prevents the process of crystallization.

Table 6. Short description of the human cell lines used in cell culture.

Cell line	Origin/Tissue/Morphology	Reference
HUH-7	Human hepatoma cell line. Adherent cells grow as monolayer	Established from surgical liver of a 57-year-old patient (NAKABAYASHI *et al.*, 1982)
HaCaT	Human keratinocyte cell line. Adherent cells grow as monolayer	Spontaneous immortal human keratinocytes (BOUKAMP *et al.*, 1988)
SAEC 6043	Human lung cell line. Adherent cells grow as monolayer	Established from surgical lower airways of a 54-year-old female patient. Company Cellsystems, number Cc-2547
RPMI 2650	Human nasal septum squamous cell carcinoma. Cells grow as monolayer	From pleural effusion of a 52-year-old man, DSMZ-Deutsch GmbH
HS-1	Human normal testis. Adherent cells grow as monolayer	Male Caucasian. ECACC
EPLC-272H	Human epidermoid lung carcinoma. Adherent cells grow as monolayer	Established from surgical lung specimens of a 54-year-old patient, DSMZ-Deutsch GmbH
TE671	Human rhabdomyosarcoma. Adherent cells grow as monolayer	Muscle cells from medulloblastoma, DSMZ-Deutsch GmbH
A549	Human lung carcinoma. Adherent cells grow as monolayer	Established from an explanted lung tumor removed from a Caucasian man in 1972
HEK 293	Human embryonic kidney. Adherent cells grow as monolayer	Cell biochemistry clinic institute neurology. Hamburg. Germany
NCI 417	Small cellular bronchial carcinoma, suspension cell line	ATCC number: CRL 5809

Table 7. Companies and their corresponding products used in cell culture.

Medium and additional cell culture material	Company and catalog number
Dulbecco´s Modified Eagle Medium (DMEM), 4.5 g/l glucose	Life Technologies, 41965-039
HAM F12	Life Technologies, 21765-029
RPMI 1640	Life Technologies, 31870-025
BEGM bullet kit	Clonetics, CC-3170
SAGM bullet kit	Cell Cystems CC-3118
Fetal calf serum (FCS)	BioWhittaker, 14-701F
Ultroser G	Life Technologies, 15950-017
PBS Dulbecco´s	Life Technologies, 14190-094
Trypsin/EDTA (1 ×)	Life Technologies, 25300-054
10.000 µg/ml Penicillin/Streptomycin (100 ×)	Life Technologies, 15140-130
200 mM (100 ×) L-Glutamine	Life Technologies, 25030-024
75 cm² cell culture flask with filter	Sarstedt, 83.1813.002
6-well cell culture plate, ∅ 35 mm	Corning Costar, 3516

Table 8. Cell lines and their corresponding culture medium

Cell line	Culture medium
HaCaT	RPMI 1640, 100 µg/ml Penicillin/Streptomycin, 2 mM L-Glutamine, 10% FCS
HUH-7	DMEM, 100 µg/ml Penicillin/Streptomycin, 2 mM L-Glutamine, 10% FCS
NCI-N 417	RPMI 1640, 100 µg/ml Penicillin/Streptomycin, 2 mM L-Glutamine, 10% FCS
A 549	10% Dulbecco´s MEM/10% FCS
RPMI-2650	DMEM (with Earle´s salts) /10% FCS/ 1 × non-essential amino acids
TE-671	80-85% DMEM/15-20% FCS
EPLC-272H	80% RPMI 1640/20% FCS
HS-1	DMEM/2 mM Glutamine/10% FCS
HEK-293	DMEM, 100 µg/ml Penicillin/Streptomycin, 2 mM L-Glutamine, 10% FCS
SAEC 6043	SAGM bullet kit

2.4.2.2 Cell stimulation

To investigate an *in vitro* inducing transcription for the new β-defensin genes, different human cell lines were incubated for 24 h with proinflammatory mediators. Cells were split on a 6-well culture dish. After 24 h, the culture reached 60-70% confluence. Following the appropriate stimulation times, cells were washed once with PBS. The cells were stimulated in three wells in parallel. The incubation with culture medium served as negative control.

Table 9. Description of the stimuli used in cell culture.

Stimuli	Company	Final concentration	Cat. number
Interleukin 1α	TEBU GmbH	20 ng/ml medium	016200-01A
Interferon γ	TEBU GmbH	40 ng/ml medium	016300-02
Lipopolysaccharide (*E.coli*)	Sigma	10 µg/ml medium	L-2654
Phorbol-12-myristate-13-acetate	Sigma	10 ng/ml medium	P-8139
TNF-α	TEBU GmbH	20 ng/ml medium	016300-01A

With the exception of Interferon γ (PBS), Interleukin 1α (100 mm trichloroethylene, 100 mM NaCl, pH 8.0) and PMA (DMSO), the rest of the proinflammatory mediators were disolved in water (ACS degrees, Sigma).

2.5 *In situ* hybridization

The study of gene products using biochemical and molecular techniques often requires tissue samples containing a considerable amount of the target molecule. However, many interesting genes are expressed either in a minority of cells in complex tissues or for only brief periods of time during the differentiation of an organism or tissue.

In order to identify the specific location and the expressing cell type of the novel human β-defensins in the epididymal tract, *in situ* hybridization was performed on human epididymal

tissue sections. The procedure was based on the fundamentals of non-radioactive *in situ* hybridization using digoxigenin-11-UTP-labeled cRNA probes described previously (TSUKAMOTO *et al*., 1991).

2.5.1 Tissue preparation

Epididymal tissues (kindly provided by A. Prützmann, St. Franziskus Hospital, Mönchengladbach, Germany) were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Tissues were washed in PBS overnight at 4°C. A further dehydration of the tissues was performed as follows:

Table 10. Solutions and conditions used during the dehydration process.

Solution	Condition
50% EtOH	Movement, overnight, room temperature
70% EtOH	Movement, overnight, room temperature
80% EtOH	Movement, overnight, room temperature
90% EtOH	Movement, overnight, room temperature
96% EtOH	Movement, overnight, room temperature

After dehydration, tissues were placed in toluol, with movement, at room temperature, overnight, and subsequently embedded in Paraplast, keeping the tissues at 65°C, for 18 h. The preparation solidified by a sudden drop in temperature.

2.5.2 Preparation of paraffin sections

Paraffin sections were cut (5 µm) using a microtome (Microm. HM340. Heidelberg, Germany) and spread on slides (SuperFrost Plus, Menzel-Glaeser, Germany). Then, sections were deparaffined 3 times for 7 min using Rothistol (Roth), and rehydrated as follows:

Table 11. Solutions and conditions used during the rehydratation process.

Solution	Condition
96 % EtOH	1 min, room temperature
90% EtOH	1 min, room temperature
80% EtOH	1 min, room temperature
70% EtOH	1 min, room temperature
50% EtOH	1 min, room temperature

Residual ethanol was removed by washing in PBS. Sections were incubated at 37°C for 30 min with 20 µg/ml proteinase K (Sigma, P2308). This step allows the RNA targets to be more accessible to the RNA probes. Samples were washed and put on a course of retrieval by embedding them in 0.2% glycin/PBS for 5 min. Sections were fixed in 4% PFA/0.2% glutaraldehyde and washed again in PBS.

2.5.3 Riboprobe preparation

Full-length cDNAs of ET-1, DEFB23, DEFB27, and DEF29 were cloned into the pGEM-T vector (Promega) in forward and reverse direction. The plasmids were linearized by enzymatic digestion.

Table 12. Matherial used for the linearization process.

cDNA	Amount of enzyme	Corresponding buffer
ET-1	8 µl *Not*I 150 U	React buffer 2 (Gibco BRL/Life)
DEFB23	8 µl *Not*I 150 U	React buffer 2 (Gibco BRL/Life)
DEFB27	8 µl *Pst*I 150 U	React buffer 3 (Gibco BRL/Life)
DEFB29	8 µl *Pst*I 150 U	React buffer 3 (Gibco BRL/Life)

For linearization, an additional 48 µl of water, ACS reagent (Aldrich, Germany) and 2 µl albumin BSA (20 mg/ml) (MBI Fermentas) were added. The samples were incubated at 37°C for 2 h and afterwards purified using the phenol/chloroform/isoamyl alcohol (25:24:1) extraction. After centrifugation at 8000 × g for 20 min, samples were transferred to a solution containing 50 µl 7.5 M NH_4Ac and 250 ml 100% EtOH. After 1 h at –80°C, tubes were centrifuged at 8000 × g for 20 min. Pellets were washed with 80% EtOH and centrifuged again under the same conditions. After drying the pellets, samples were resuspended in 15 µl of DEPC water. DNA was quantified by photometric analysis and adjusted in order to obtain 1 µg during the reverse transcription reaction:

Reverse transcription reaction:

1 µg linearized cDNA
2 µl 0.1 M DTT (Gibco BRL/Life)
4 µl 5 × transcription buffer (Promega)
2 µl 10 × DIG nucleotides (Roche)
1 µl RNasin 10000 U (40 U/µl)
1 µl T7 RNA-polymerase (1000 U) (Roche)

The vector pGEM-T offers the possibility to generate the riboprobes using two enzymes (T7- or SP6- RNA polymerase). Nevertheless, DIG-labeled RNA probes were generated using only T7-RNA polymerase (Roche) in order to reduce the differences in efficiency, caused by using two different enzymes. The solutions were incubated at 37°C for 2 h. After adding 2 µl DNase I (1U/µl, Life Technologies) the samples were incubated at 37°C for 10 min.

Samples were subsequently purified by precipitation with 2 µl of 0.2 M EDTA, 2.5 µl 4 M LiCl, and 75 µl 100% EtOH. Samples were kept at –80 C for 2 h. Then, they were centifuged at 8000 × g for 15 min at 4°C and washed twice with 100 µl EtOH 70%. Afterwards, the tubes were centrifuged under the same conditions and the pellet was resuspended in 50 µl of DEPC-

water. Samples were stored at –80°C for no longer than six months. The efficiency of the riboprobe production was checked by standard electrophoresis (see 2.2.5).

2.5.4 Hybridization procedure

In situ hybridization was performed on slices from human testis, caput, corpus, and cauda epididymis. Prehybridization was carried out for 1 h at 55°C in prehybridization solution. Hybridization was performed with a suitable amount of labeled riboprobes (3-5 µl, according to the results of a previous electrophoresis) in 100 µl of prehybridization solution at 55°C in a humidity chamber for 16 h. The slides were subsequently rinsed in 2 × SSC (17.5 g NaCl, 8.8 g sodium citrate, 800 ml ddH_2O) pH 4.5 for 5 min followed by two washing steps in 2 × SSC pH 4.5/50% deionized formamide at 50°C for 15 min. Sections were treated with blocking solution for 1 h at room temperature. The slides were subsequently incubated overnight at 4°C with an anti-DIG-antibody conjugated to alkaline phosphatase (Roche) diluted 1:1500 in blocking solution. The process continued with two washing steps in 0.02% Tween/PBS and equilibration in NTMT twice for 5 min.

Prehybridization solution:

50%	Deionized formamide
5 ×	SSC
1%	Blocking reagent (Boehringer)
10 mM	Maleic acid pH 7.5
15 mM	NaCl
0.1%	EDTA
0.1%	Tween 20
0.1%	CHAPS (Sigma)
0.1%	Heparin (Sigma)
1 mg/ml	Yeast tRNA

Blocking solution:

2%	Blocking reagent (Boehringer)
0.02%	Tween 20
1%	Normal sheep serum in PBS, pH 7.5

NTMT:

50 mM	Tris/HCl
100 mM	NaCl
50 mM	$MgCl_2$
0.05%	Tween 20

2.5.5 Signal detection

Detection was performed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Sigma) for 2 h at room temperature. Slides were washed, fixed in 4% paraformaldehyde and mounted in Mowiol solution.

Mowiol solution:

48 g Mowiol 488 [Sigma-Aldrich 32.459-0]

21.1% Glycerol/ 0.1 M Tris/HCl, pH 8.5

DAPI staining was performed by adding DAPI 1 µg/ml for 3 min. Subsequently slides were washed in PBS and visualized by fluorescent microscopy.

Additional controls, like omitting the probe or pretreatment with 100 mg/ml RNase A for 10 min, were also included in this study. The pretreatment with RNase A was performed to eliminate the signal produced by RNA. This would omit the positive signal.

2.6 Chemical synthesis of β-defensins

2.6.1 Solid-phase peptide synthesis

The β-defensin peptides used in this study were synthesized in the Peptide Synthesis Division of IPF PharmaCeuticals GmbH by Dr. Enno Klüver and Dr. Axel Schulz. The following part will briefly state the synthesis strategies for the defensin peptides.

All peptides were synthesized according to the principle of solid-phase peptide synthesis (MERRIFIELD *et al*., 1986) with the use of the fluorenylmethoxycarbonyl (Fmoc)-protecting group on an automated peptide synthesizer 433A (Applera). Synthesis was performed using preloaded TentaGel resins (Rapp Polymere). TG R PHB-Lys(Boc) Fmoc for hBD27, hBD23 and hBD29 with standard HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate)/HOBt (1-hydroxybenzotriazol) activation in N-methylpyrrolidinone on a scale of 0.1 mmol. All cysteine residues were trt-protected. The resulting peptidyl resins were treated at room temperature with a mixture of trifluoracetic acid / ethanedithiol / water (94:3:3, v/v/v, 40 ml/g resin) for 3-4.5 h and then precipitated by filtration into ice cold tert-butylmethylether (TBME). The resulting products were separated by centrifugation, washed with TBME and dried under vacuum. The crude peptides were dissolved in diluted acetic acid and loaded onto a preparative Vydac C18 column (47 × 300 mm, 15 - 20 µm, flow rate 40 ml/min; solvent A, 0.07% TFA; solvent B, 0.07% TFA in acetonitrile/H_2O 80:20; UV detection at 215 nm; with the following gradient: 20-60% B in 80 min). The fractions containing the desired

peptides, as detected by mass spectrometry and analytical C18 HPLC, were pooled and lyophilized. hBD23 and hBD29 were used in their linear forms, whereas hBD27 was folded using a 0.1 M NH_4HCO_3 buffer containing 2 mM EDTA, 1.6 mM cysteine, 0.8 mM cystine at pH 8 at 4°C under a N_2-atmosphere for 25 h. The folded peptide was purified using a preparative Vydac C18 column (47 × 300 mm, 15-20 µm, flow rate 40 ml/min; solvent A, 0.07% TFA; solvent B, 0.07% TFA in acetonitrile/H_2O 80:20; UV detection at 215 nm; with a gradient of 30-55% B in 50 min). The relative molecular masses (Mr) were determined by ESMS (Sciex API III, Perkin-Elmer): hBD27 (folded): 4751.3 (Mr calc. 4750.5), hBD23 (linear): 4523.9 (Mr calc. 4523.3), hBD29 (linear): 4249.6 (Mr calc. 4250.1). hBD4 (folded) 4367.1 (Mr calc. 4366.1).

2.6.2 Assignment of the disulfide pattern of hBD27

The disulfide pattern of hBD27 was determined by two independent methods. Proteolytic digestion with thermolysin and identification of the obtained fragments by mass spectrometry and stepwise Edman degradation revealed a disulfide bond between Cys2 and Cys4. A fragment, containing Cys1, Cys3, Cys5 and Cys6, was identified, but it could not be decided whether Cys1 is connected to Cys5 or Cys6 (and, respectively, Cys3 to Cys6 or Cys5). In another approach, hBD27 was partially reduced using an established method (GRAY *et al.*, 1993). The free thiol groups of the partially reduced hBD27 were alkylated and the amino acid sequence of the product was determined by automated Edman degradation. The result allowed the assignment of disulfide bond Cys3-Cys6. Thus, synthetic hBD27 shows the disulfide pattern Cys1-Cys5, Cys2-Cys4, Cys3-Cys6, which is identical to the pattern predicted for β-defensins (TANG *et al.*, 1993).

2.6.3 Assignment of the disulfide pattern of hBD4

In order to determine the disulfide pattern of synthetic hBD4, a sample of the peptide was subjected to proteolytic digestion with trypsin (modified, sequencing grade, Boehringer Mannheim, Germany) and chymotrypsin (sequencing grade, Boehringer Mannheim, Germany) at 36°C for 4 h. The combination of these two proteases allows cleavage of the peptide backbone between the cysteine residues. This method is well established and widely used in the assigment of disulfide patterns. The fragments, which were obtained from the digestion of hBD4, were separated by HPLC and the collected fractions were first analyzed by electrospray mass spectometry. The fractions which correspond to fragments containing cysteine residues were subjected to automated Edman degradation on a 494 protein sequencer (ABI) in order to confirm the amino acid sequence. Thus, two fragments could be identified, the first containing the fragment Cys4-Pro-Asn-Thr-Tyr, connected to a fragment Cys-Arg, the second containing the

three fragments Cys1-Ile-Gly Tyr, Cys-Arg and Ala-Cys5-Cys6-Leu-Arg. This analysis left two questions open: the origin of the Cys-Arg fragments (one of them containing Cys2, the other Cys3) and the connectivity of the two adjacent cysteine residues. In order to solve this problem, another proteolytic digestion was performed with the combination of endoproteinase Arg-C and chymotrypsin (both sequencing grade, Boehringer Mannheim, Germany) at 36°C for 5 h. The digestion products obtained were separated and identified as described above. One fraction contained the fragments Glu-Leu-Asp-Arg-Ile-Cys1-Gly-Tyr, Cys3-Arg-Ser-Gln-Glu-Tyr and Ala-Cys5-Cys6-Leu, confirming the connection of Cys1 and Cys3 to Cys5/Cys6. Furthermore, the Edman degradation of this peptide gave the possibility to solve the second problem, concerning the disulfide connectivity of the two adjacent cysteine residues, since it allows the Cys-Cys peptide bond to be cleaved, which is not possible by proteolytic digestion. The Pth-derivates of cysteine, which are products of every degradation cycle, are analyzed by HPLC. Derivates of cysteine, which appear after the second cysteine of a disulfide bridge has cleaved, could be detected in the third and sixth degradation cycle, respectively. The cysteine in the third cycle could generate only from the cysteines Cys3 and Cys6. Thus, the entire disulfide array is as follows: Cys1-Cys5, Cys2-Cys4, Cys3-Cys6. This is in full agreement with the disulfide pattern, which is published for the bovine BNBD-12 and which is regarded to be characteristic for β-defensins.

2.7 Monoclonal antibody production

Monoclonal, hBD-4-specific antibodies were generated by Dr. Aleksandra Heitland. Mice were immunized five times for a period of 17 days (short-term immunization: day 1, 4, 7, 14, and 17) with coupled hBD4. For the immunization process, 200 µg antigen were required for one mouse (10 animals were used for immunization, because the immune response depends on the interindividual variability). Low molecular weight peptides (e.g. defensins) are not usually immunogenic. The immunogenicity was enhanced by gentle polymerization with a carrier of proteins (hemocyanin). The antigen was covalently bound to carbodiimide via the N-terminus. The chemical coupling provides a better immunological response of the IgG-carbodiimide complexes. Subsequently, popliteal lymph nodes of the immunized mice were fused (NIEBUHR *et al*., 1998) with X63-Ag8.653 tumor cells. Thereby, spleen cells from an immunized mouse are fused with mutated myeloma cells lacking the enzyme HPRT. Cells were grown in a medium containing hypoxanthine, aminopterin and thymidine (HAT). Unfused myeloma cells cannot grow in this medium because aminopterin blocks the purine synthesis. B cells die in this medium even though they contain an enzyme (HPRT) that would allow them to utilize the hypoxanthine

placed in the medium. The fused cells (hybridomas) grow and divide. The supernatant of wells in which the hybridomas grow are screened by direct ELISA for the presence of reactive monoclonal antibodies. The hybridomas producing this antibody are grown and frozen (YELTON and SCHARFF, 1980). The selectivity of the resulted antibodies were tested by Western blot. The antibodies were used during the immunohistochemical studies.

2.8 Characterization of antibodies by Western blot

To test the selectivity and sensitivity of the antibodies used in immunohistochemistry, Western blot was performed under reducing conditions. Prior to the electrophoresis, a denaturation of the samples and a prestained protein marker, Mark 12 Unstained Standard (Invitrogen), was performed at 70°C for 10 min.

Samples were applied to the gel which was incorporated into an equipment XCell Sure Lock gel chamber (Invitrogen). To obtain the sharpest bands, NuPAGE reducing agent, 0.5 M DTT in stabilized liquid form (Invitrogen), was added just prior to heating and loading the samples. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (NP0323, Invitrogen) with a separation range of 2.5-200 kDa was used to achieve the separation. Denaturing running buffer, 2-N-morpholinoethanesulfonic acid NuPAGE MES SDS (Invitrogen), filled the inner chamber and partially the outer one. Then, 500 µl of reducing agent NuPAGE (NP0005, Invitrogen) were added to the running buffer in the inner chamber. This ensured that the reduced samples did not reoxidize during electrophoresis. This undesirable effect could result in band broadening or band splitting while the proteins migrate down the gel. The electrophoresis was performed at 200 V for 35 min.. Subsequently, the electrophorized NuPAGE gels were removed, washed, and prepared for a further blotting process which was performed using a transference chamber XCell II Blot Module (Invitrogen). The PVDF transfer membrane (0.2 µM pore size, NEN), and filter paper were embedded in methanol and transfer buffer respectively for an efficient transfer. The transfer system was set up by assembling the blotting pads and gel-membrane sandwich horizontally in the cathode (-) core of the blot module in the same sequence, so that the gel was closest to the cathode plate. After adding enough presoaked blotting pads to rise to 0.5 cm over the rim of the cathode core, anode (+) core was placed on the top of the pads. The blot module was filled with transfer buffer until the gel-membrane sandwich was covered. The transference was performed at 30 V for 60 min.

Running buffer:

50 ml	20 × NuPAGE running buffer (NP0002)
950 ml	ddH_2O

Transfer buffer:

50 ml	20 × NuPAGE transfer buffer (NP0006-1)
1 ml	NuPAGE Antioxidants
200 ml	Methanol
749 ml	ddH_2O

After transference, the membrane was washed three times using TBS-T. The membrane was blocked with 1 × Roti-Block (Roth) at room temperature for 1 h, to avoid non-specific binding sites. Membrane was incubated with 4.5 ml of anti-hBD4 antibody and 500 µl of 10 × Roti-Block (Roth) at 4°C, overnight. Afterwards, the membrane was washed three times with TBS-T. Subsequently, the membrane was incubated with secondary antibody (anti-mouse-IgG peroxidase conjugate) (Sigma, A9917), diluted 1:50.000 in 1 × Roti-Block at room temperature for 1 h and shaken at 1.500 rpm. The membrane was washed 5 times with TBS-T. The signal was developed using SuperSignal West Dura, Extended Duration Substrate (Pierce). This product is a sensitive enhanced chemiluminescent substrate for detecting horseradish peroxidase (HRP) on immunoblots. Signal was detected using the photographic method. Thus, CL-XPosure film (Pierce) was exposed for 5 min and developed using Agfa Rodinal (1:25). The reaction was stopped and results were fixed using Agfa Tetenal Superfix I (1:7). Membrane with the corresponding peptides was stained with Indian ink to determine equal sample loading.

10 × TBS (Tris-saline buffer):

24.2 g	Tris
80.0 g	NaCl

The pH value of the solution was adjusted to pH 7.6 with approximately 20 ml of 25%HCl, and afterwards filled up with ddH_2O until 1000 ml.

1 × TBS-Tween:

100 ml	10 × TBS
0.5 ml	Tween 20
900 ml	ddH_2O

Indian ink solution:

50 ml	TBS-T
0.15 ml	Fount Indian ink (Pelikan)

2.8.1 Study of selectivity of monoclonal antibodies

An analysis of the crossreactivity was performed. For every synthetic peptide, (hBD1, hBD2, hBD3, hBD4) 100 ng/µl were applied to a denaturing polyacrylamide gel. After blotting, an anti-hBD4 monoclonal antibody was applied on a membrane containing the mentioned electroblotted

peptides. Subsequently, the results of the blotting process were developed as previously described.

Antibodies included in this study: L1310D1; L1310D2; L1310G5; L1310G6; L1310H10; L1310A12; L11135aD12.

2.8.2 Study of sensitivity of monoclonal antibodies

To study the sensitivity of the antibodies, serial concentrations (3 ng, 30 ng, 100 ng, 300 ng) of hBD4 (cyclic form) were applied to an electrophoresis gel (SDS-PAGE) (NP0323, Invtrogen). Anti-hBD4 monoclonal antibody was applied to the membrane containing the synthetic peptide. Subsequently, the results of the blotting process were developed as previously described (see 2.8).

2.9 Immunohistochemistry

Immunohistochemistry is a technique that detects antigens in tissue sections and cells by lectins, monospecific monoclonal or polyclonal antibodies. In the present work, immunohistochemistry was based on the use of enzyme-linked antibodies to detect tissue antigens. The substrate is converted by the enzyme into a colored product that precipitates on the slide at the site of the reaction (CHILDS and UNABIA, 1982).

2.9.1 Tissue preparation

The tissue used in immunohistochemistry was the tissue previously used for *in situ* hybridization (see 2.5.1).

2.9.2 Preparation of paraffin sections

Sections were cut (5 µm) using a microtome (Microm HM340. Heidelberg, Germany) and spread on slides (SuperFrost Plus, Menzel-Glaeser, Germany). Afterwards, they were deparaffined using Rothistol (Roth). Slides were rehydrated 3 times for 7 min (Table 13).

Table 13. Solutions and conditions used during the rehydration process.

Solution	Condition
96% EtOH	2 min, twice
90% EtOH	2 min
80% EtOH	2 min
70% EtOH	2 min
50% EtOH	2 min
ddH_2O	3 min
PBS	3 min

An antigen retrieval process was then performed on the sections. Thus, 4.7 ml of unmasking solution (Vector Laboratories) were diluted in water to a final volume of 500 ml. Sections were boiled for 3 min, and chilled to 30ºC. This step was repeated twice. Subsequently, sections were washed in PBS for 8 min. Endogenous peroxidases may generate an intrinsic and non-specific signal. These enzymes were inactivated by 3% H_2O_2, for 15 min. Slices were washed in PBS-T (0.2% Tween 20) twice for 4 min. Sections were treated with blocking solution (PBS-T+ 10% FCS) for 1 h to avoid non-specific bindings of the first antibody. Slides were incubated with primary undiluted supernatant (containing anti-hBD4 monoclonal antibody), at 4ºC, overnight. Endothelin-1 (ET-1) was used as positive control. ET-1 monoclonal antibody (Oncogenes, CN Biosciences (UK) LTD, cat No. CP44-100) was used to identify the antigen. Then, sections were washed in PBS-T, three times for 5 min. A biotinylated secondary antimouse antibody was used to detect the primary antibody. One drop (±50 µl) of this biotinylated secondary antibody (Vectastain ABC kit Elite, Mouse IgG, Vector Laboratories) was diluted in 2.5 ml of PBS-T. This solution was supplemented with one drop (±50 µl) of normal serum. Slices were washed for 5 min. Preparations were incubated with ABC vectastain reagent (avidin biotinylated horseradish peroxidase macromolecular complex), for 30 min at room temperature. Therefore, the binding complex was amplified by adding this preformed molecule. Avidin is a glycoprotein with a high affinity for the small molecular weight vitamin, biotin. The binding of avidin to biotin is essentially irreversible. To remove the excess of these macromolecular complexes, sections were then washed with PBS-T, twice for 2 min. The signal was developed by adding staining solution for 3 min:

1 ml PBS
one tablet urea-H_2O_2, Sigma fast
one tablet 3.3´diaminobenzidine, Sigma fast

Slices were mounted using Mowiol solution (48 g Mowiol 488 [Sigma-Aldrich, Cat. 32.459-0] in 21.1% glycerol/ 0.1 M Tris/HCl, pH 8.5).

2.10 Antimicrobial activity

2.10.1 Radial diffusion assay

Radial diffusion assay (LEHRER *et al*., 1991) is a fast and simple procedure for the examination of antimicrobial activity in aqueous solutions. It consists of the measurement of the inhibition of bacterial growth, surrounding an applied substance in feeding agar. Bacteria strains were grown in 5 ml of growth medium at 37°C for 18 h, until they reached a $OD_{600} = 0.8$ (see 2.4.1). Subsequently, 500 µl of the bacteria were diluted in 100 ml of the suitable medium (see 2.4.1). Then, 25 ml medium were placed into sterile Petri dishes, which were stored at 4°C. With the help of a sterile hollow needle, small holes were punched (3 mm in diameter) and removed from the gel. The peptides were applied in these cavities in different concentrations (2 µg, 10 µg, 20 µg). The agarose plates were then incubated at 37°C for 18 h. The positive controls, 10 µg of doxycycline, vancomycin, and MBI 28 were applied in cavities on the same plates. The diameter of the inhibition zone around the wells was measured using the metric scale (0.1 mm increments). The diameter of clearing was expressed in units (0.1 mm=1 U) and was calculated by subtraction of the well diameter (3 mm = 30 U).

0.03%	Tryptic Soy Broth (Sigma T-8261)
0.80%	NuSieve GTG Agarose (Biozym)
0.02%	Tween 20
10 mM	Natriumphosphate buffer, pH 7.2

2.10.2 Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) is the lowest concentration (µg/ml) of an antibiotic that inhibits bacterial growth under standard conditions. The MIC for a bacterial population is determined by a serial dilution test (SÜßMUTH *et al*., 1999). MIC and the agar diffusion test (see 2.10.1) are optimal methods to evaluate the antimicrobial activity. Series of test tubes were filled with different concentrations of a particular antibiotic. Serial dilutions of peptides (factor 0.5) were prepared in test medium, TSB in 10 mM sodium phosphate buffer (pH 7.2). First of all, 100 µl of a defined concentration of peptide was applied in test medium in the superior cavity, while all other recesses were filled by adding 50 µl pure test medium. Starting at the superior cavity, 50 µl solution containing peptide were transferred to the underlying cavities in each case, mixed and this solution of bisected concentration was transferred again to a cavity below. Bacteria grew for 18 ± 2 h and then a standard inoculum of 50 µl bacterial suspension in test medium containing 2×10^5 to 5×10^5 CFU/ml was added to each test tube with 50 µl of the prepared peptide solution. After incubation at 37°C for 16 to 20 h, bacterial growth was determined by visual analysis and

absorbance measurement at 570 nm using a Dynatech microplate reader. Since the number of added cells has a direct influence on the MIC value, the dilution of the overnight culture of bacteria has to be determined previously.

The dilution level went from 1:2000 to 1:10.000 according to the NCCLS guidelines (ISENBERG *et al*., 1993). The germ density determined experimentally was the result of a 1:10000 dilution from the original culture. The optimal number of bacteria in this dilution is $2\text{-}5 \times 10^5$. A further 1:1000 dilution was performed and 100 µl were spread onto an agar plate. The plate was incubated overnight. Then, colonies were counted and the value was multiplied by 10.000 in order to know the number of bacteria per milliliter of a 1:10000 dilution.

2.11 Hemolysis studies

The investigation of the hemolytic activity of the new human β-defensins was achieved according to the studies of HELMERHORST *et al*., 1999. Erythrocytes from collected full blood from healthy young donors was stored in citrate-buffered (Monovetten, Sarstedt). Blood was then centrifuged at $1500 \times g$ for 10 min and the supernatants removed with a Pasteur pipette. The erythrocyte-rich pellet was washed, resuspended in one volume of test medium (1/10 TSB, 287 mM glucose, PH 7.4), and centrifuged as previously. The isotonic glucose concentration was used to prevent a nonspecific hemolysis in the hypotonic environment (osmoprotection). Erythrocytes were diluted with test medium to a final volume of 200 µl. On the other hand, different concentrations of hBD23 and hBD27 (1-250 µg/ml) were pipetted and lyophilized into a 96-well-micro V bottom plate. The erythrocyte solution was added to the wells containing different concentrations of hBD23 or hBD27 and incubated at 37°C for 1 h. The 96-well-micro V bottom plates were centrifuged afterwards at $1000 \times g$ for 5 min. Then, 150 µl of supernatant per cavity was transferred into a new 96-well-micro V bottom plate with a flat botton. The solution was colored by free hemoglobin and the concentration determined by measurement at $\lambda = 450$ nm in a microplate reader (BIO-Tech Instruments). The incubation with different concentrations of MBI-28 (GOUGH *et al*., 1996) was used as reference for the hemolytic activity. Erythrocytes were incubated with test medium which was used as negative control.

The hemolytic activity of hBD-4 was calculated according to the following formula:

$$\text{Hemolysis [\%]} = \frac{\text{Ø } A^{450nm} \text{ (hBDX) - Ø } A^{450nm} \text{ (Negative control)}}{\text{Ø } A^{450nm} \text{ (1\% Tween 20) - Ø } A^{450nm} \text{ (Negative control)}} \text{ x100}$$

2.12 Data bank and software

The comparison of different sequences of DNA fragments as well as the translation of these nucleotide sequences into an amino acid sequence were achieved with the Sequencer V3.1 software (ABI).

The search for public and accessible sequence information (e.g. EST clones, genomic contigs) was performed in Internet data bases: NCBI (National Centers for Biotechnology Information) and its algorithm basic local alignment search tool (BLAST, ALTSCHUL *et al.*, 1997). The program signalP V2.0 was used for the investigation of putative signal peptide sequences (NIELSEN *et al.*, 1997). This thesis was created with programs of the Microsoft Office package. The graphics, including media and standard deviations (SD), represented in the results section, were calculated with GraphPad Prism 3.0.

2.13 Discovery of new β-defensin genes *in silico*

Biosciences are progressively dependent on information technology and comprise a huge amount of complex data. Genes and DNA have attracted most attention, since DNA sequencing techniques have allowed genome sequencing projects to be a reality. Since the beginning of the systematic human genome analysis, a great deal of effort has been made to identify new genes by using bioinformatical approaches. During the present work several bioinformatical programs were used.

BLAST (Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA.

ORF Finder (Open Reading Frame Finder) is a graphic analysis tool which is designed to find all open reading frames of a selectable minimum size in a user's sequence or in a sequence already in the database. This tool identifies open reading frames using the standard or alternative genetic codes. The deduced amino acid sequence can be saved in various formats and searched against the sequence database using the BLAST server.

PATTINPROT is a tool to scan a protein database of one or several sequences for one or several patterns.

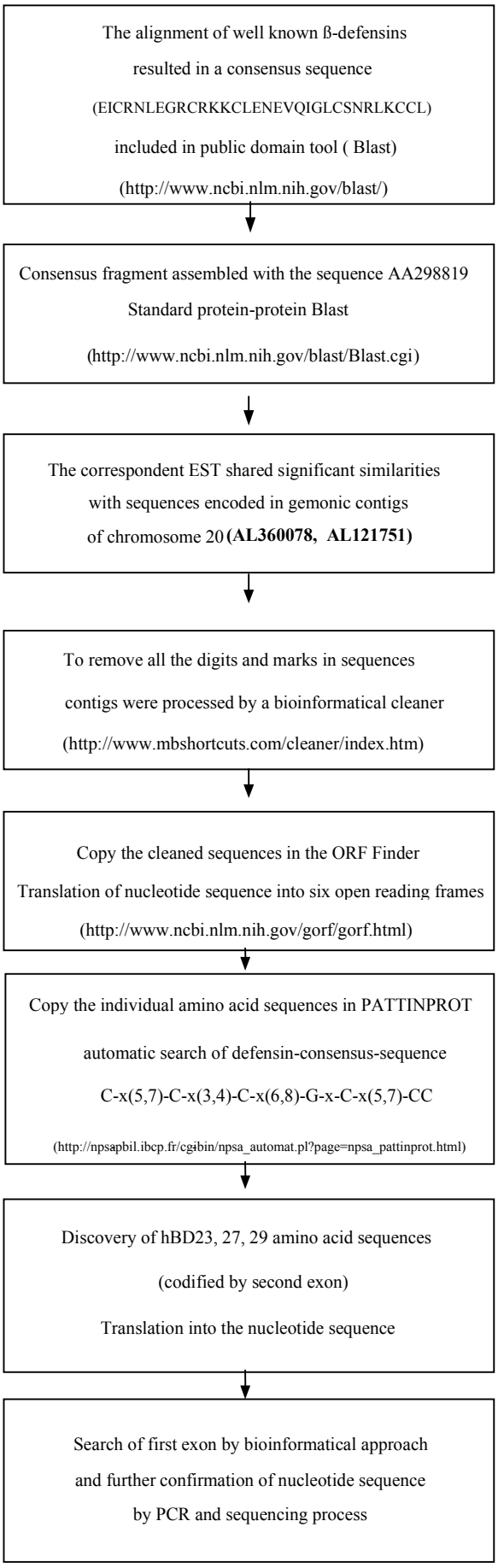


Figure 11. Strategy for the identification of new β-defensin genes.
The discovery of these new genes was based on the use of bioinformatical approaches described in the diagram.

3. Results

3.1 Identification of full-length β-defensin cDNAs

The identification and description of three new genes was based on the fundamentals of functional genomics. The systematic search for new human β-defensin genes as previously described (Figure 11) led to the identification of three new genes. The alignment of known β-defensin amino acid sequences mapping on chromosome 8 (hBD1 to hBD4) resulted in a consensus sequence (EICRNLEGRCRKKCLENEVQIGLCSNRLKCCL), which was included in a public domain tool (BLAST). This bioinformatical approach assembled the consensus fragment with a sequence (AA298819) whose corresponding EST sequence shared significant similarities to sequences located on two genomic contigs, at the telomere (AL360078) and centromere (AL121751) of chromosome 20. The complete sequences were translated into six open reading frames. The different frames were translated into the amino acid sequence and analyzed for the occurrence of the β-defensin-specific cysteine pattern using PATTINPROT (http://npsa-pbil.ibcp.fr). The well known cysteine pattern for human β-defensins was found in three sequences. Thereby, three new members of this family of cationic peptides were identified (DEFB23, DEFB27, and DEFB29). After deducing the putative second exon of the genes, their full-length cDNAs were investigated. For this purpose the research was performed as follows:

1-Search by computer of the putative first exon: upstream sequences from the second exon were translated *in silico* into three frames. Then, the sequences were analyzed using SignalP server (http://www.cbs.dtu.dk/services/SignalP-2.0/) to identify their putative signal peptides.

2-For each candidate sequence (deduced previously *in silico*), reverse primers from the second exon and forward primers upstream of a putative ATG start codon were designed and tested for amplification. Using human testis cDNA as a template, specific products were detected, subsequently cloned into pGEM-T vector (Promega) and sequenced. In addition, during the bioinformatical analysis of chromosome 20, two putative genes were found (DEFB16 and DEFB22). These sequences were not considered as real genes due to the following reasons:

An iterative blast search was performed against the human databases to identify additional related sequences and a search for EST sequences that would confirm the transcription of the sequences. However, no evidence was found.

To confirm whether or not these sequences are expressed, RACE-PCR was accomplished under different conditions and several tissues (testis, skeletal muscle, liver, lung) were tested for the occurrence of the two genes. These experiments gave no amplification product, leading to the

conclusion that previous exons with the corresponding start codons do not exist for these sequences.

3.2 Structure of DEFB23, DEFB27, and DEFB29 genes

The analysis of the genomic structure revealed that all newly identified sequences consist of only two exons and one intron. After the translation of DNA to RNA, this molecule needs to be matured by elimination of the intron during the splicing process.

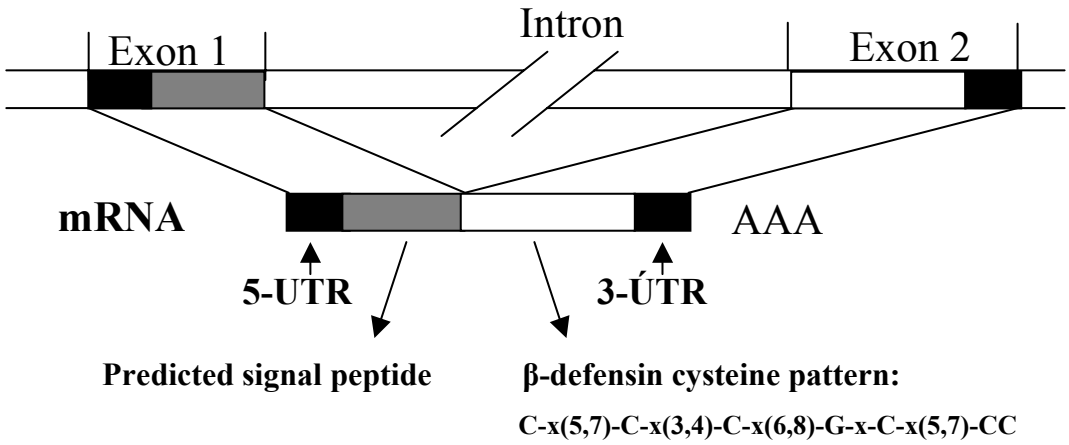


Figure 12. Schematic representation of the common genomic structure of DEFB23, DEFB27 and DEFB29 genes.
The genes consist of two exons separated by the intron. The intron is subsequently eliminated during the maturation of RNA. Untranslated regions (UTR) at the 5´and 3´ends as well as the poly-A tail are also indicated. The predicted signal peptide is encoded by the first exon and the β-defensin cysteine pattern by the second exon.

This genomic structure was also shared by DEFB1, DEFB2, DEFB3, DEFB4, and DEFB6 whereas DEFB5 exhibited 3 exons (YAMAGUCHI *et al*., 2002). The analysis showed that a predicted signal peptide is encoded by a first exon and the cysteine-containing core structure which is indicative of the β-defensin family is encoded by the second exon. Further PCR analysis and *in silico* EST search provided no indication of additional exons or splicing variants for any of the novel cDNAs. After the study of the sequences *in silico* and further confirmation in the laboratory, more details were revealed about the composition of these three new genes, as shown in the following figures (Figure 13, Figure 14, Figure 15). The signal peptide is also depicted, indicating the putative position for the cleavage site of the amino acid sequences that would result in the mature peptide.

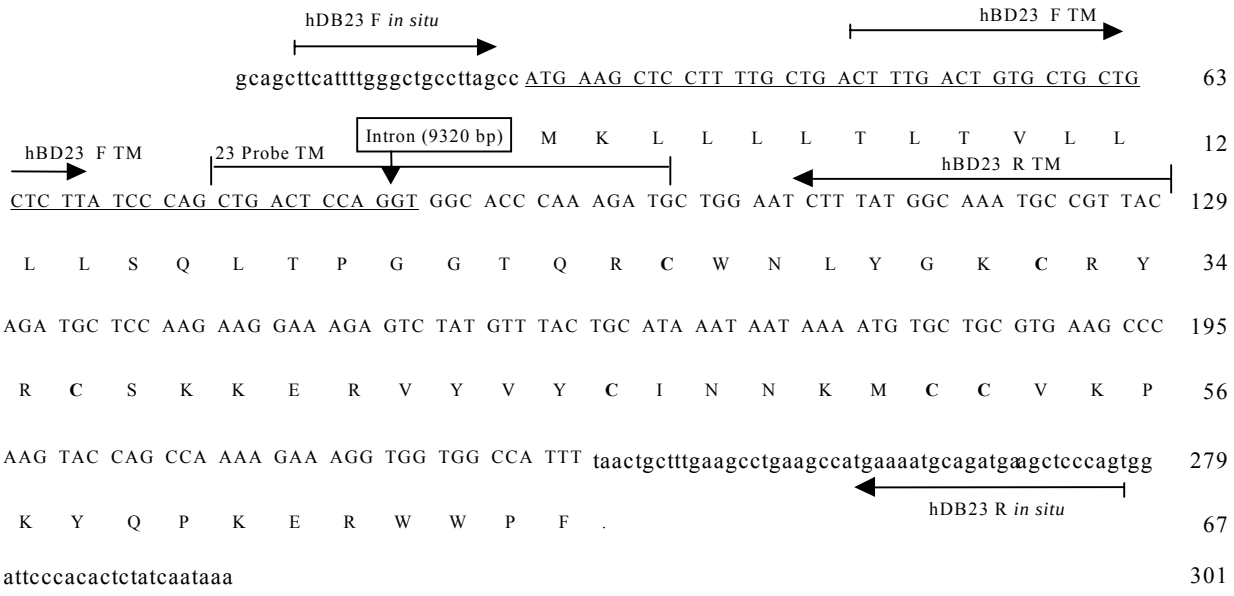


Figure 13. Structure of the hBD23 gene.
The translated amino acid sequence is positioned under the cDNA sequence. Capital letters correspond to the translated region. The cDNA encoding the putative signal peptide is underlined. The intron is labeled by a vertical arrow separating exon 1 and exon 2. The characteristic six cysteines of β-defensins are labeled with bold letters. Position and orientation of all oligonucleotides used for cloning, study of expression, and regulation analysis are indicated by horizontal arrows.

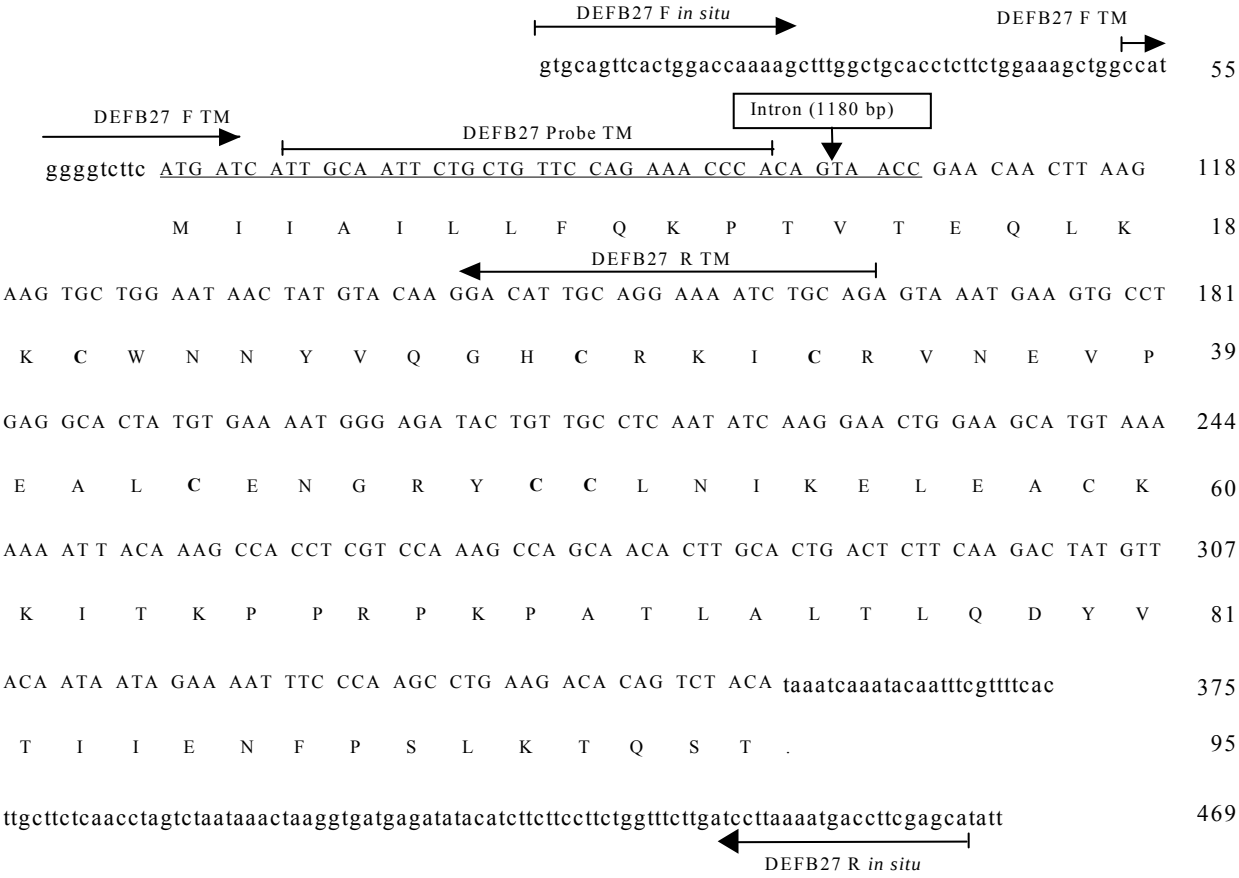
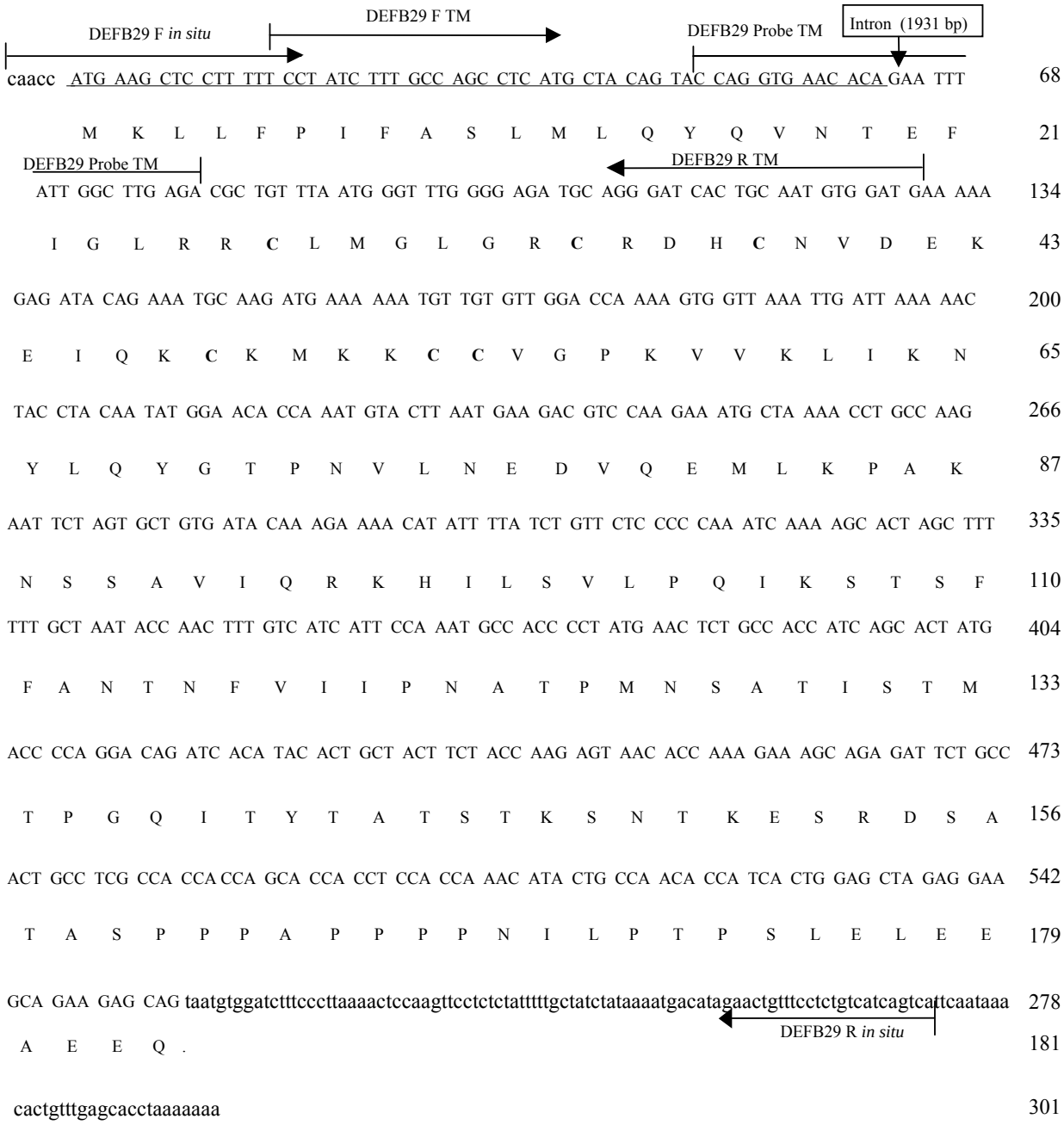


Figure 14. Structure of the hBD27 gene.
The translated amino acid sequence is positioned under the cDNA sequence. Capital letters correspond to the translated region. The cDNA encoding the putative signal peptide is underlined. The intron is labeled by a vertical arrow separating exon 1 and exon 2. The characteristic six cysteines of β-defensins are labeled with bold letters. Position and orientation of all oligonucleotides used for cloning, study of expression, and regulation analysis are indicated by horizontal arrows. The gene was registered in Gene Bank with the accession number AF525929.



caacc ATG AAG CTC CTT TTT CCT ATC TTT GCC AGC CTC ATG CTA CAG TAC CAG GTG AAC ACA GAA TTT 68

M K L L F P I F A S L M L Q Y Q V N T E F 21

ATT GGC TTG AGA CGC TGT TTA ATG GGT TTG GGG AGA TGC AGG GAT CAC TGC AAT GTG GAT GAA AAA 134

I G L R R **C** L M G L G R **C** R D H **C** N V D E K 43

GAG ATA CAG AAA TGC AAG ATG AAA AAA TGT TGT GTT GGA CCA AAA GTG GTT AAA TTG ATT AAA AAC 200

E I Q K **C** K M K K **C** **C** V G P K V V K L I K N 65

TAC CTA CAA TAT GGA ACA CCA AAT GTA CTT AAT GAA GAC GTC CAA GAA ATG CTA AAA CCT GCC AAG 266

Y L Q Y G T P N V L N E D V Q E M L K P A K 87

AAT TCT AGT GCT GTG ATA CAA AGA AAA CAT ATT TTA TCT GTT CTC CCC CAA ATC AAA AGC ACT AGC TTT 335

N S S A V I Q R K H I L S V L P Q I K S T S F 110

TTT GCT AAT ACC AAC TTT GTC ATC ATT CCA AAT GCC ACC CCT ATG AAC TCT GCC ACC ATC AGC ACT ATG 404

F A N T N F V I I P N A T P M N S A T I S T M 133

ACC CCA GGA CAG ATC ACA TAC ACT GCT ACT TCT ACC AAG AGT AAC ACC AAA GAA AGC AGA GAT TCT GCC 473

T P G Q I T Y T A T S T K S N T K E S R D S A 156

ACT GCC TCG CCA CCA CCA GCA CCA CCT CCA CCA AAC ATA CTG CCA ACA CCA TCA CTG GAG CTA GAG GAA 542

T A S P P P A P P P P N I L P T P S L E L E E 179

GCA GAA GAG CAG taatgtggatctttcccttaaaactccaagttcctctctatttttgctatctataaaatgacatagaactgtttcctctgtcatcagtcattcaataaa 278

A E E Q . 181

cactgtttgagcacctaaaaaaa 301

Figure 15. Structure of hBD29 gene.
The translated amino acid sequence is positioned under the cDNA sequence. Capital letters correspond to the translated region. The cDNA encoding the putative signal peptide is underlined. The intron is labeled by a vertical arrow separating exon 1 and exon 2. The characteristic six cysteines of β-defensins are labeled with bold letters. Position and orientation of all oligonucleotides used for cloning, study of expression, and regulation analysis are indicated by horizontal arrows. The gene was registered in Gene Bank with the accession number AF525931.

3.3 Comparison of exon-intron boundary region

The corresponding exon-intron boundaries were assessed by alignment of the cDNAs with the genomic contig. Conserved splicing sequences were detected inside exon 1, exon 2, and the intron within the regions close to the exon-intron boundaries.

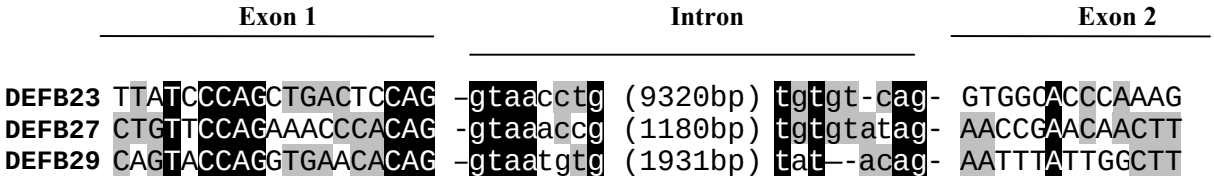


Figure 16. Comparative study of the exon-intron boundary region for DEFB23, DEFB27, and DEFB29.
The numbers in parentheses indicate the length of the introns. Short but conserved sequences for the three new genes were detected and marked in black and those conserved in only two genes are labeled in grey.

3.4 Chromosomal location

Defensins described so far (DEFB1, DEFB2, DEFB3, DEFB4, DEFB5, and DEFB6) have been located on chromosome 8p23. In contrast, the three new defensins described in the present work are mapping on chromosome 20, separated in two different clusters: DEFB23 on the centromere of chromosome 20 (20q11.1), whereas DEFB27 and DEFB29 on the telomere of this chromosome (20p13).

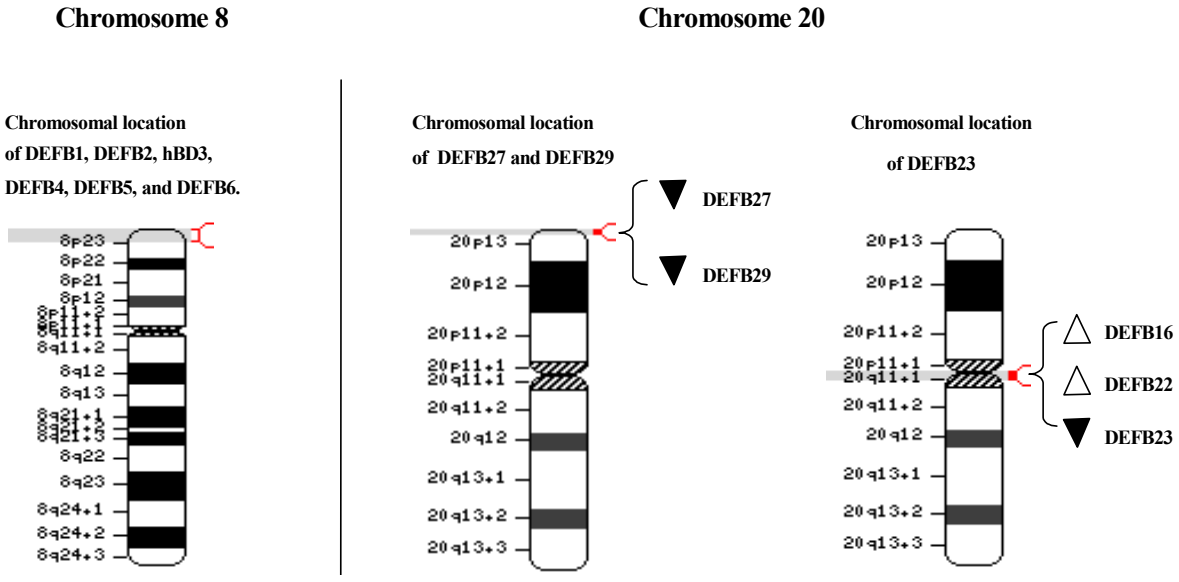


Figure 17. Chromosomal location for β-defensin genes.
Mapping regions and the orientation of the new genes on chromosome 20 are indicated (black arrow). The pseudogenes DEFB16 and DEFB22 are also depicted (open arrow).

During the analysis of chromosome 20, two candidate pseudogenes were found: DEFB16 and DEFB22. These genes are located on chromosome 20q11.1 and positioned in the opposite direction to the existing β-defensins.

The analysis and comparison of the introns for eight β-defensins did not allow a direct correlation between their length and the chromosomal location. Nevertheless, the difference between the intron length of DEFB3 and DEFB23 was notable. DEFB5 was not included in the table since this β-defensin exhibited two introns.

Table 14. Length of introns of β-defensins described to date.

	Chromosome 8				Chromosome 20			
	DEFB1	DEFB2	DEFB3	DEFB4	DEFB6	DEFB23	DEFB27	DEFB29
Intron	6972 bp	1692 bp	943 bp	4495 bp	3575 bp	9320 bp	1180 bp	1931 bp

The length of hBD1 (6972 bp) was determined by alignment of the genomic DNA (accession number AF205406) with the cDNA (accession number NM005218) by using the program Pairwise Blast 2 sequences (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). This length differed from the length proposed by Kaiser and Diamond (2000) that was reported to be 10 kb.

3.5 Comparison of amino acid sequences

Using the full-length cDNA as template, the deduced amino acid sequences were aligned in conjunction with the sequences of known β-defensins and β-defensin-like proteins. A comparison between amino acid sequences encoded by genes mapping on chromosome 8 and those encoded on chromosome 20 was performed. According to the results obtained *in silico* with the signal peptide program, the predicted signal peptide for β-defensins could be obtained. The described exon for pseudogene DEFB16 coded for an amino acid sequence containing seven cysteines, a property only shared by the confirmed DEFB5, DEFB6, and DEFB27 genes. The additional cysteine of hBD16 and hBD5 was located in a position preceding the cysteine-containing core, whereas for hBD6 and hBD27 it occurred behind the mentioned pattern.

Two common features, a conserved glycine between cysteines 1 and 2, as well as a glutamic acid between cysteines 3 and 4 were observed. In contrast to the primary structure of the published peptides hBD1 to hBD6, HE2C, HE2E (Chr. 8p23) and hBD23 (Chr. 20q11.1), longer carboxy-terminal extensions following the cysteine-containing core were determined for hBD27 and hBD29 (both on Chr. 20p13). The mentioned terminal fragments exhibited only minor sequence similarities within themselves or any other known protein motif.

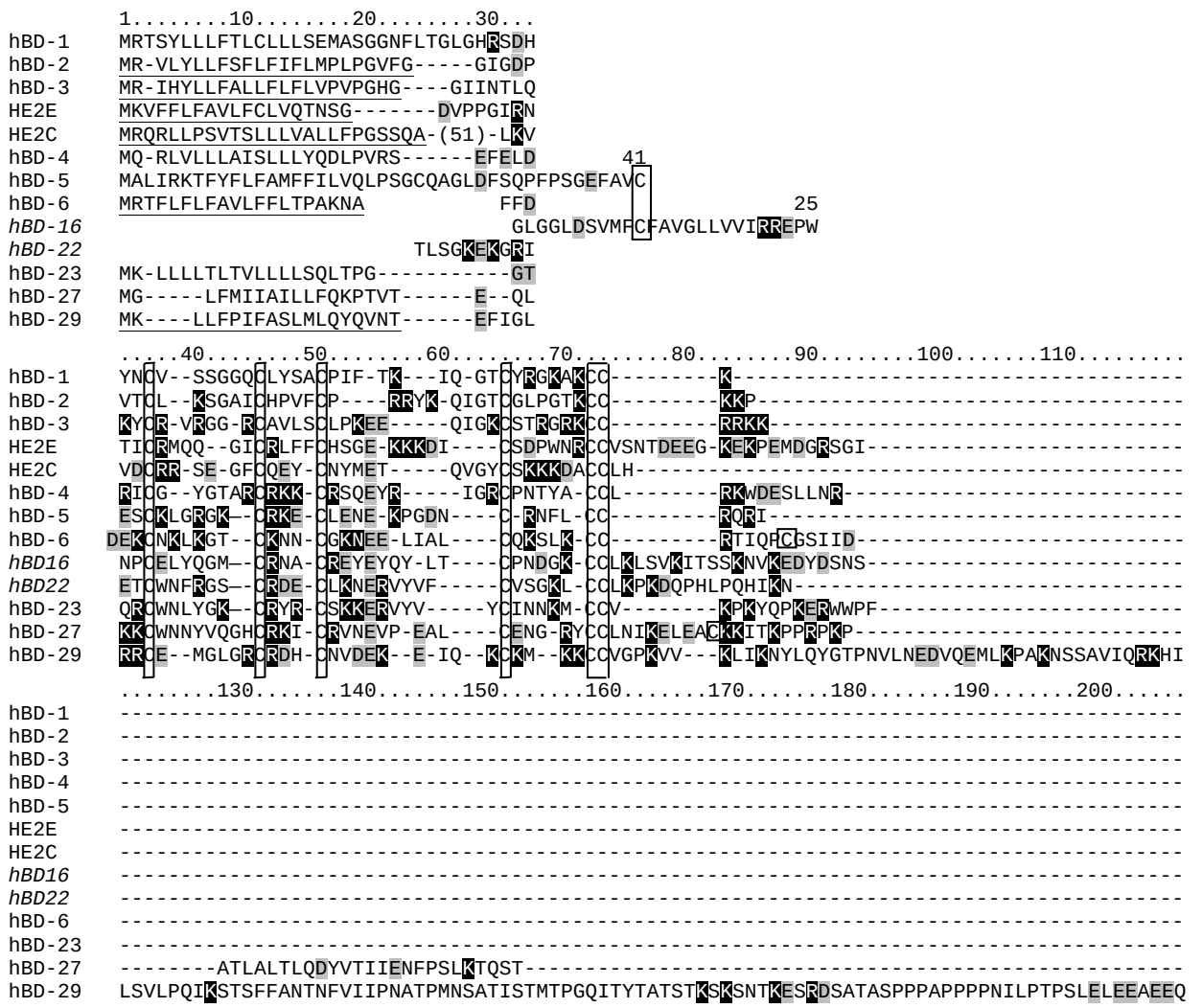


Figure 18. Multiple sequence alignment of putative human β-defensin peptides.
The predicted signal peptides are underlined. The conserved and additional cysteines are boxed. Acidic amino acids are shaded in gray, whereas basic residues are printed inversely. The number in parentheses denotes additional amino acids at the amino-terminus of HE2C. Pseudogenes are labeled in italics.

3.6 Endogenous expression of the new β-defensin genes in human tissues

The tissues were purchased from Clontech Laboratories (Palo Alto, CA. USA) or kindly provided by Professor Dr. Markus Buechler (Heidelberg, Germany) and PD Dr. Helmut Friess (Bern, Switzerland) (see 2.3.1). The mRNA abundance in 28 human tissues was quantified including known places of β-defensin expression.

After the isolation of mRNA, a standard procedure of reverse transcription was carried out (see 2.3.3). cDNA was produced from each tissue and used for the determination of expression of each new gene.

Following the cloning, a systematic approach for the evaluation of the relative transcript level in 28 human tissues was performed by real-time quantitative RT-PCR (*TaqMan*) (see 2.3.4). Using the Primer-Express Software (Perkin-Elmer), specific forward primers (TM.F), reverse primers (TM.R), and double-labeled *TaqMan* probes (TM.P) were deduced for DEFB23, DEFB27, DEFB29.

The length of the resulting amplicons was designed to be between 87 and 115 bp. The amplicons are shorter than the full-length cDNA in order to reduce the possibility that the enzyme leaves the synthesis during the extension process before reaching the position of the reporter.

In order to increase specificity, only amplicons spanning the exon-intron boundary of the corresponding β-defensin gene were chosen. Therefore, a highly comparable efficiency of amplification was provided.

Serial dilutions of the cloned cDNAs were used to generate a standard curve. Amplifications of all tissues were normalized to these standard curves leading to a relative copy number of the corresponding DEFB transcripts.

For relative quantification, these data were normalized to glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) expression levels in each tissue tested. Experiments were performed three times in duplicate. An absolute standard curve used as external calibration required that the absolute quantities of the standard were known by some independent means. Plasmid DNA with the insert of interest served as absolute standard and was used for sample interpolation. Thus, calculations were performed applying a described mathematical formula (see 2.3.5). Using this approach, the transcription level of the new β-defensins in different human tissues was compared.

The amplification in some tissues was significant, but had a range of 1 to 25 transcripts in 7.5 ng RNA-equivalent amount with a high standard deviation, therefore they were not included in the following figures.

The study of DEFB23 expression showed a restricted pattern located in five tissues. The highest expression of DEFB23 was found in the male reproductive tract. Constitutive expression was also detected in skeletal muscle, trachea, heart and lung. The relative abundance of transcripts of DEFB23 is much higher in testis and skeletal muscle than in trachea, heart and lung.

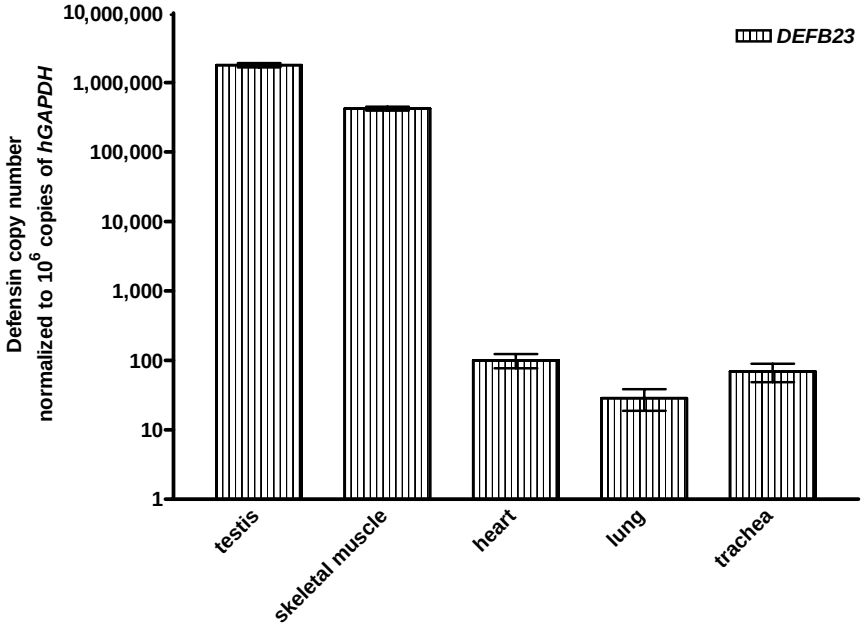


Figure 19. Tissue distribution of DEFB23.
The expression pattern of DEFB23 was studied by real-time quantitative RT-PCR, (*TaqMan*) in 28 different human tissues. The remaining tissues are not shown because some of them had a range of 1 to 25 transcripts in 7.5 ng RNA-equivalent amount with a high standard deviation. The relative abundance was normalized to hGAPDH (+/-SD in logarithmic scale). Experiments were carried out at least twice in duplicate.

The evaluation of the expression pattern of DEFB27 showed that this gene was expressed in testis, kidney, pancreas, skeletal muscle, liver, and lung. DEFB27 had a more widespread distribution in the human body. Consistent with the previously studied DEFB23, the highest expression level was detected in the male reproductive organ.

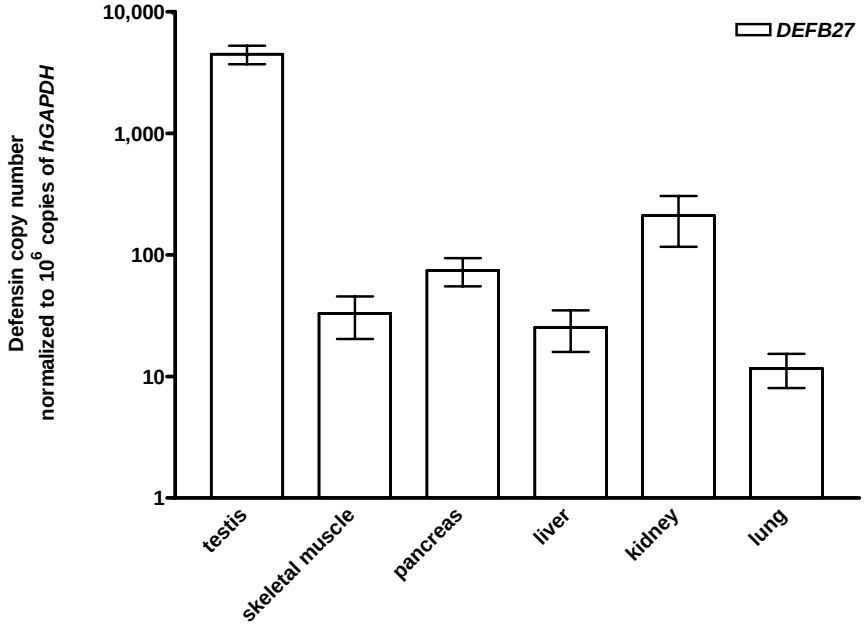


Figure 20. Tissue distribution of DEFB27.
The expression pattern of DEFB27 was studied by real-time quantitative RT-PCR, (*TaqMan*) in 28 different human tissues. The remaining tissues are not shown because some of them had a range of 1 to 25 transcripts in 7.5 ng RNA-equivalent amount with a high standard deviation. The relative abundance was normalized to hGAPDH expression (+/- SD in logarithmic scale). Experiments were performed at least twice in duplicate.

Whereas other tissues like trachea (DEFB23; HONG *et al.*, 2002; DIAMOND *et al.*, 2000), or skin (CHRONELL *et al.*, 2001; BOS *et al.*, 2001; HUH *et al.*, 2002; ONG *et al.*, 2002; MARCHINI *et al.*, 2002) show expression of known β-defensins, DEFB27 expression is completely absent in these tissues.

The study of hBD29 expression in human tissues revealed a restricted distribution in the male genital tract and skeletal muscle, showing an exclusive expression for these two tissues. No detectable expression was found in any of the other tissues tested.

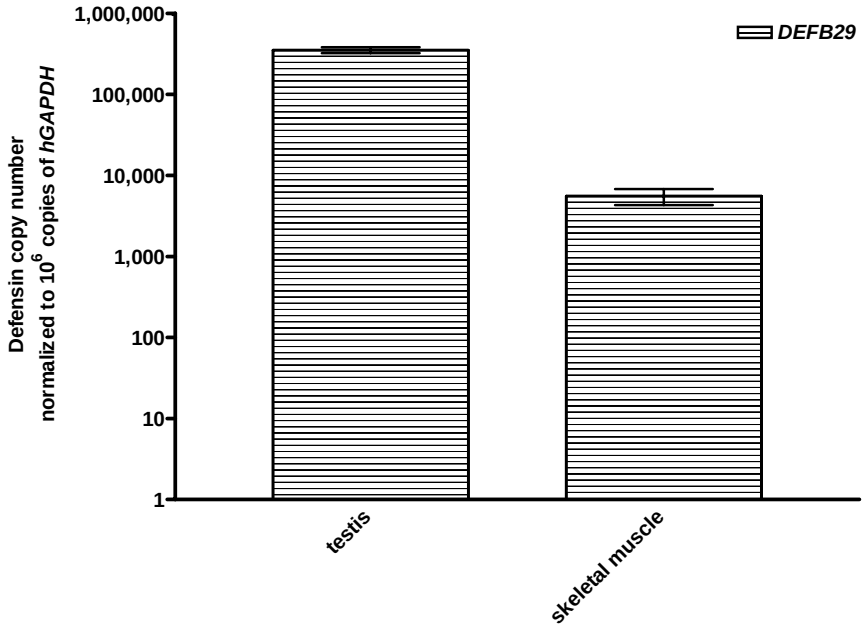


Figure 21. Tissue distribution of DEFB29.
The expression pattern of DEFB29 was studied by real-time quantitative RT-PCR, (*TaqMan*) in 28 different human tissues. The remaining tissues are not shown because some of them had a range of 1 to 25 transcripts in 7.5 ng RNA-equivalent amount with a high standard deviation. The relative abundance was normalized to hGAPDH (+/-SD in logarithmic scale). Experiments were performed at least twice in duplicate.

A comparison of the expression for the three genes (DEFB23, DEFB27, and DEFB29) was performed. The tissues that showed constitutive expression of the new β-defensin genes are shown (Figure 22). DEFB27 was expressed in more tissues than the other two β-defensins. In fact, DEFB29 was specifically expressed in two tissues, testis and skeletal muscle. In general, both tissues had a high number of copies for the three genes. DEFB27 was expressed in liver, pancreas and kidney, whereas the other two new β-defensin genes were not expressed. DEFB23 was expressed in trachea, a frequently infected tissue, but no transcripts were detected for DEFB27 and DEFB29.

Therefore, each defensin exhibited a distinct expression pattern. However, a common feature seemed to be the high number of copies of the three new genes in testis and skeletal muscle.

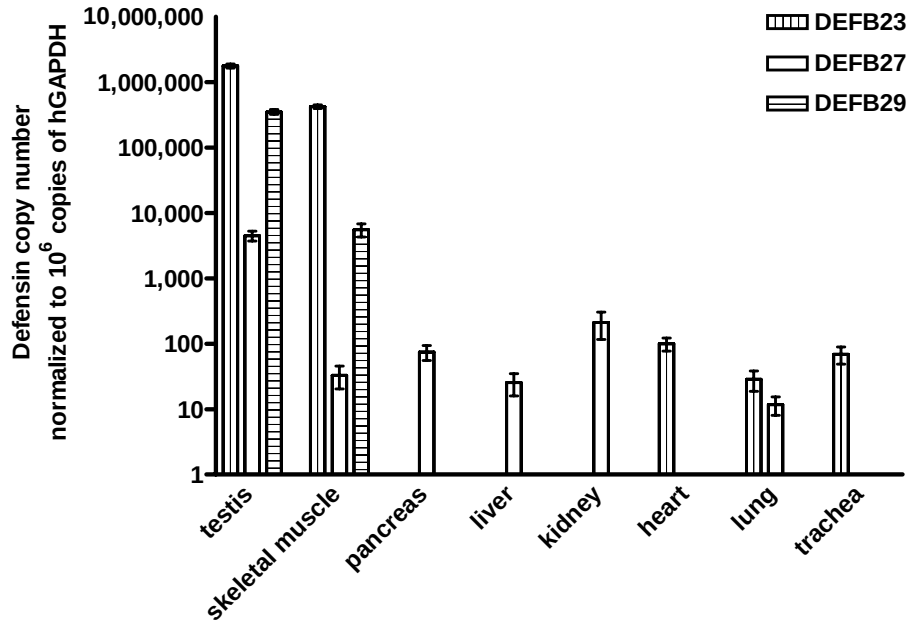


Figure 22. Comparison of tissue distribution for the novel DEFB23, DEFB27, and DEFB29 genes.
Tissue distribution of DEFB23, hBD27, and hBD29 studied by real-time quantitative RT-PCR (*TaqMan*). The remaining tissues are not shown because some of them had a range of 1 to 25 transcripts in 7.5 ng RNA-equivalent amount with a high standard deviation. Relative abundance was normalized to hGAPDH (+/-SD in logarithmic scale). Experiments were performed at least twice in duplicate.

3.7 Regional distribution of the new β-defensin genes in human epididymis

Since the male genital tract exhibited the highest copy number, the interest became focused on this organ. The local distribution of the three novel β-defensins in testis and epididymis was studied in more detailed research. The expression of the previously described DEFB4, which exhibited a comparable tissue distribution pattern, with the highest mRNA abundance in the male genital organ (GARCIA *et al.*, 2001-B; RODRIGUEZ-JIMENEZ *et al.*, 2003) was also evaluated. For this purpose, tissue samples were obtained from four different patients suffering from testicular tumors. Tissues were kindly provided by Prof. Dr. C. Stief from the Urology Department of Hannover Medical School (MHH, Hannover, Germany). The tissue was separated into six functionally different parts (testis, rete testis, caput, corpus, the proximal and distal segments of cauda epididymis). RNA was isolated to generate the corresponding cDNAs. Significant interindividual differences in the expression pattern of the four patients were not observed. An illustrative picture above each figure was added for better visualization of the anatomical distribution (e.g. Figure 23, modified from the Sobotta Atlas of Anatomy, URBAN and SCHWARZENBERG 1997).

3.7.1 Study of DEFB23 expression along the male reproductive tract

The evaluation of the DEFB23 expression in different segments of human testis and epididymis, showed than in general the highest level of expression was in testis. It is interesting to note that the transcription level decreased from testis to rete testis, but increased again at the beginning of the epididymal tract. The analysis of expression within this tissue reflected that caput epididymis exhibited the highest copy number for DEFB23, but the number of transcripts decreased in the more distal parts of the duct. Figure 23 shows a comparison of DEFB23 expression among the four different patients.

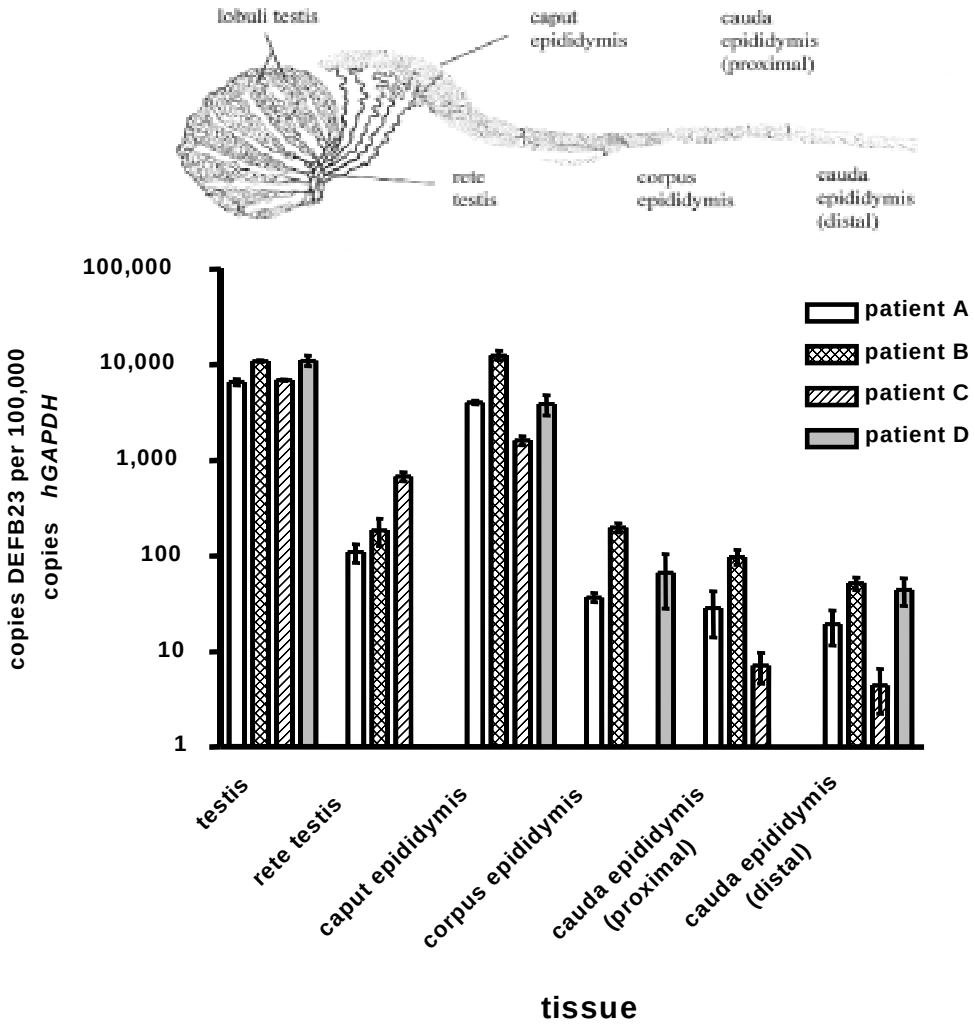


Figure 23. Regional distribution of DEFB23 mRNA in different segments of human epididymis.
Total RNA from six different parts of epididymis from four different patients was isolated. After cDNA synthesis, real-time quantitative RT-PCR (*TaqMan*) was carried out for each epididymal part. The copy number of transcripts was normalized to hGAPDH (+/- SD in logarithmic scale).

3.7.2 Study of DEFB27 expression along the male reproductive duct

The study of expression for DEFB27 in the male reproductive organ revealed that the relative abundance of transcripts was much higher in epididymis than in the testicular tissues. This distribution of expression contrasted with the expression pattern of the previously described DEFB23. High constitutive expression along all parts of the epididymis peaking in the midpart (corpus) was detected for DEFB27. Figure 24 shows a comparison of DEFB27 expression among four different patients.

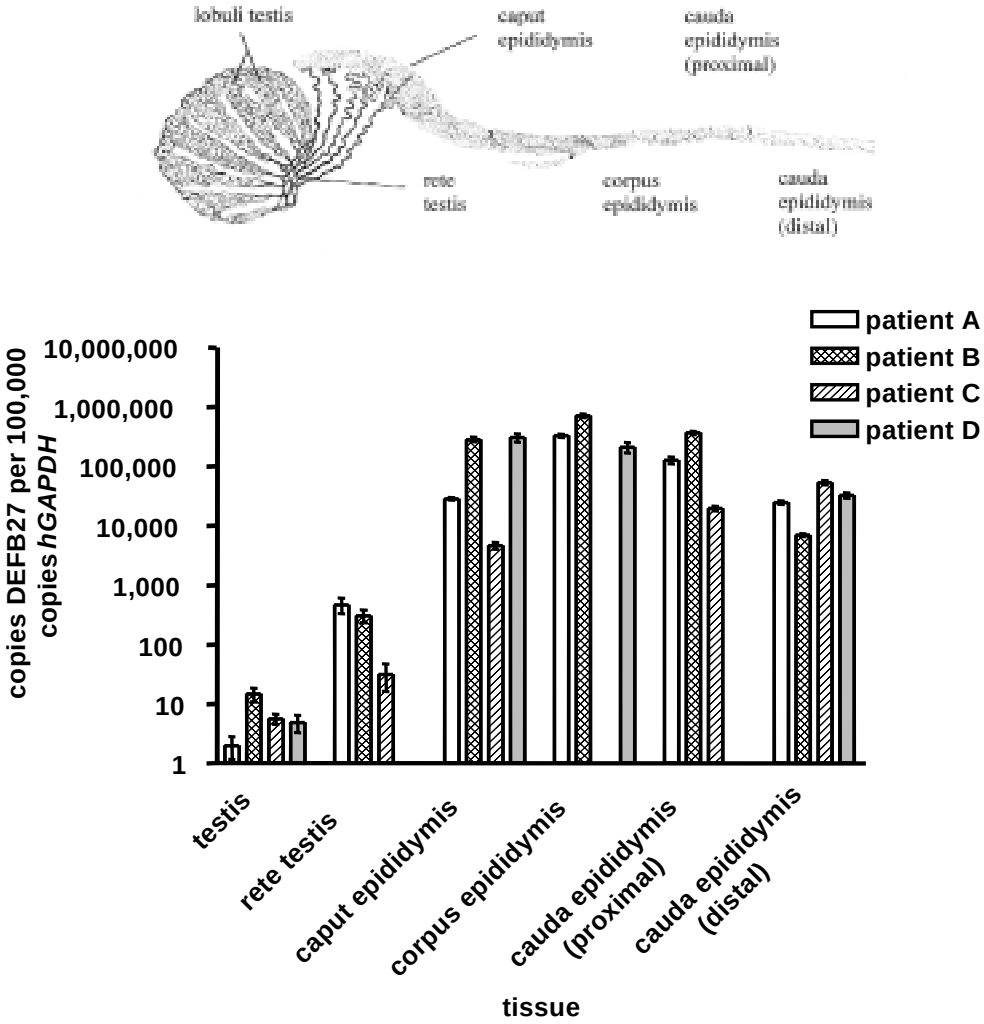


Figure 24. Regional distribution of DEFB27 mRNA in different segments of human epididymis.
Total RNA from six different parts of epididymis from four different patients was isolated. After cDNA synthesis, real-time quantitative RT-PCR (*TaqMan*) was carried out for each epididymal part. The copy number of transcripts was normalized to hGAPDH (+/- SD in logarithmic scale).

3.7.3 Study of DEFB29 expression along the male reproductive duct

DEFB29 together with DEFB27 exhibited a similar regional distribution in the male reproductive tract. These two genes shared a similar chromosomal location, both mapping on the same cluster, to be precise on the telomere of chromosome 20 (20p13). The relative abundance of copies for DEFB29 was higher in epididymis than in testicular tissues. The general number of transcripts for DEFB27 and DEFB29 along epididymis reached a higher level in comparison to DEFB23 encoded on the centromere (20q11.1). Figure 25 shows a comparison of the expression of DEFB29 among four different patients.

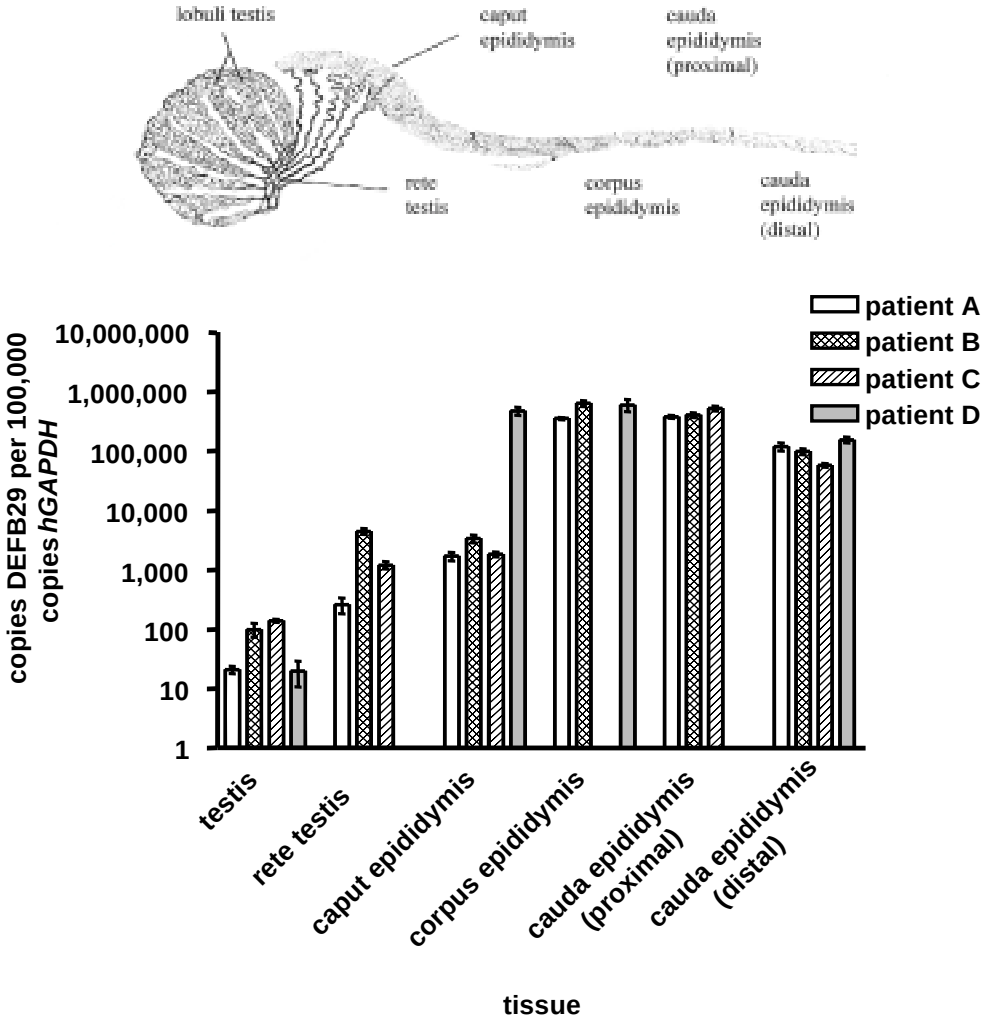


Figure 25. Regional distribution of DEFB29 mRNA in different segments of human epididymis.
Total RNA from six different parts of epididymis from four different patients was isolated. After cDNA synthesis, real-time quantitative RT-PCR (*TaqMan*) was carried out for each epididymal part. Copy number of transcripts was normalized to hGAPDH (+/- SD in logarithmic scale).

3.7.4 Expression profile of the novel genes and DEFB4 along the genital tract

A comparison of the profiles for the three novel β-defensin genes, together with the previously described DEFB4 (RODRIGUEZ-JIMENEZ *et al.*, 2003), revealed that DEFB4 exhibited a comparable expression pattern, with the highest number of transcripts in the epididymal tract. On the other hand, the relative mRNA abundance for DEFB4 and DEFB23 in epididymis was lower than for DEFB27 and DEFB29. The highest copy number for DEFB27 and DEFB29 was detected in corpus epididymis. In contrast DEFB23 mRNA was approximately one thousand-fold less abundant in this epididymal region.

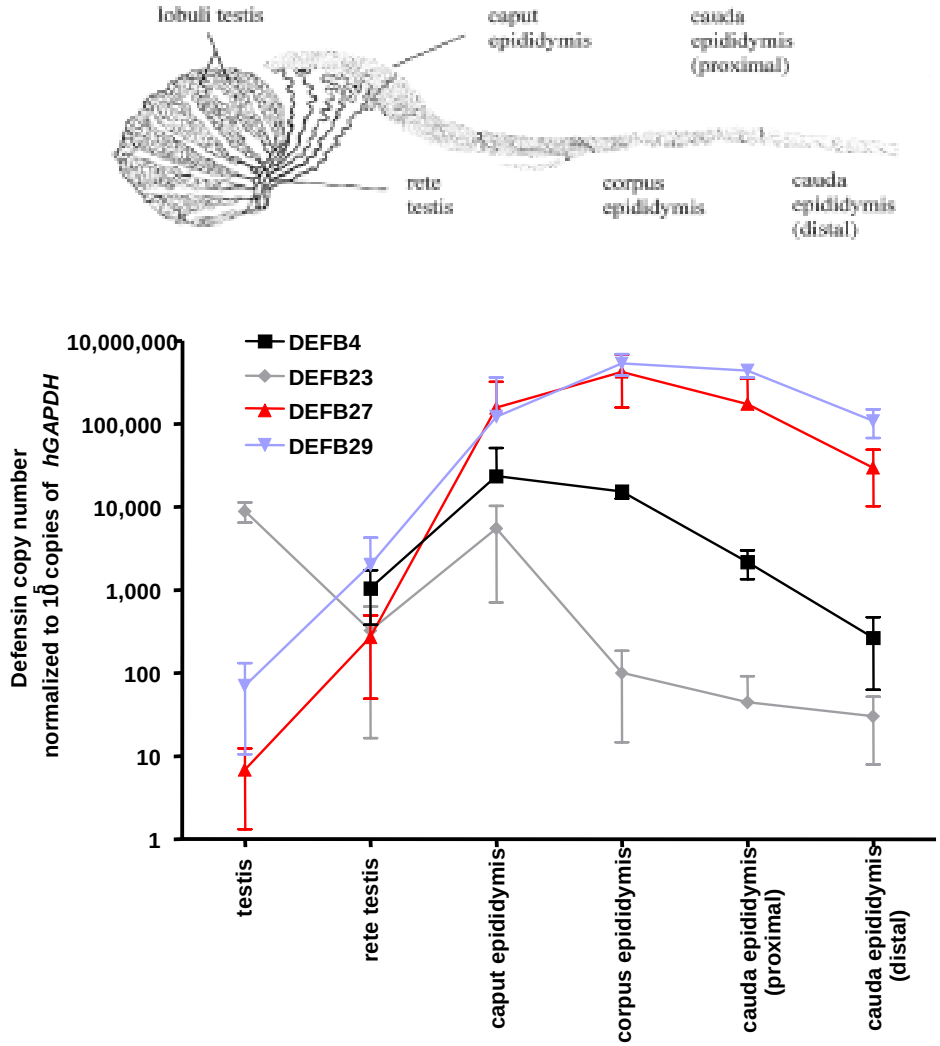


Figure 26. Comparison of regional distribution for DEFB23, DEFB27, DEFB29 and the previously described DEFB4 in different segments of human epididymis.
Total RNA from six different parts of epididymis from four different patients was isolated. After cDNA synthesis, real-time quantitative RT-PCR (*TaqMan*) was carried out for each epididymal subunit. Copy number of transcripts was normalized to hGAPDH (+/- SD in logarithmic scale).

All in all, the tendency of expression within the epididymal tract for DEFB4 and DEFB23 was different to DEFB27 and DEFB29. It was shown that DEFB23 copy number decreased along the more distal parts of the epididymal tract (Figure 26). The number of transcripts of DEFB27 and

DEFB29 increased from caput to corpus epididymis although a slight reduction of expression was also detected in the region of cauda epididymis. A notable difference of copies between DEFB23 and DEFB29 in the distal cauda epididymis could be observed (Figure 26).

3.8 Studies on the regulation of DEFB23, DEFB27, and DEFB29

β-defensins have been found to be either constitutively expressed or induced by infection and inflammation. Cell lines were stimulated with five inflammatory factors. After stimulation, RNA was extracted and reverse transcribed to the corresponding cDNAs. The study was performed by real-time quantitative RT-PCR (*TaqMan*). Eight stimulated cell lines from different organs were studied, including: (HS-1, human normal testis) and (TE-671, human rhabdomyosarcoma) obtained from testis and skeletal muscle respectively, tissues with high expression for the β-defensins studied here. Some of the cell lines were included in the study since they showed regulation for other previously described β-defensin genes. For instance, DEFB1 and DEFB2 (SEO *et al.*, 2001) and DEFB3 (GARCIA *et al.*, 2001-A) in HaCaT (keratinocyte cell line), DEFB4 in HEK293 (human embryonic kidney cells) and SAEC6043 (human small epithelial cells), (GARCIA *et al.*, 2001-B).

A549 (human lung carcinoma cells), EPLC (human epidermoid lung carcinoma), NCI-417 (small cellular bronchial carcinoma) were also included in the study. These cell lines were obtained from tissues which are frequently colonized by pathogenic germs and β-defensins act as antimicrobial agents (BALS *et al.*, 1998). In each case, expression was undetectable or at the very limit of detection. The number of transcripts was never higher than 25 copies but with a high standard deviation.

Table 15. Regulation study of DEFB23, DEFB27, and DEFB29. Cells were stimulated with five different inflammatory factors.

Inflamatory factors:

TNF-α = [20 ng/ml]
LPS = [10μg/ml]
INTγ = [40 ng/ml]
PMA = [1μM]
IL-1α = [20ng/ml]

	Control	TNF-α	PMA	INT-gamma	LPS	IL-1
HS-1	0	0	0	0	0	0
NCI-417	0	0	0	0	0	0
HEK-293	0	(1-25) DEFB23	0	0	0	0
SAEC-6043	0	(1-25) DEFB29	(1-25) DEFB29	0	0	0
HACAT	0	0	0	0	0	0
EPLC	0	0	0	0	(1-25) DEFB29	(1-25) DEFB29
A549	(1-25) DEFB23	0	0	0	0	0
TE-671	(1-25) DEFB27	0	0	0	0	0

In addition, the expression of the three genes was also studied in two unstimulated cell lines (HUH-7, RPMI2650). However, these cells did not show any basal expression for the new genes. This study led to the conclusion that there was neither significant basal expression nor regulation in the cell lines tested.

3.9 Cellular localization in human testis and epididymis

To identify the exact location and the cell types that are responsible for the novel human β-defensin gene expression in the epididymal tract, *in situ* hybridization was performed. The experiments were carried out on testicular and epididymal sections. For this purpose, the full-length cDNA of endothelin-1 (ET-1) (used as positive control), DEFB23, DEFB27, and DEFB29 were cloned into pGEM-T vector. The cDNA sequences were inserted into the vector in forward and reverse direction in order to generate sense and antisense probes. The plasmids were linearized using suitable restriction enzymes (Figure 27, A).

ET-1 and DEFB27 were cut using *Not* I, whereas DEFB23 and DEFB29 were linearized by *Pst* I. After linearization, DIG-labeled RNA probes were generated using T7-RNA polymerase. Efficiency of the *in vitro* transcription reaction was estimated by standard electrophoresis (2% agarose gel), (Figure 27, B).

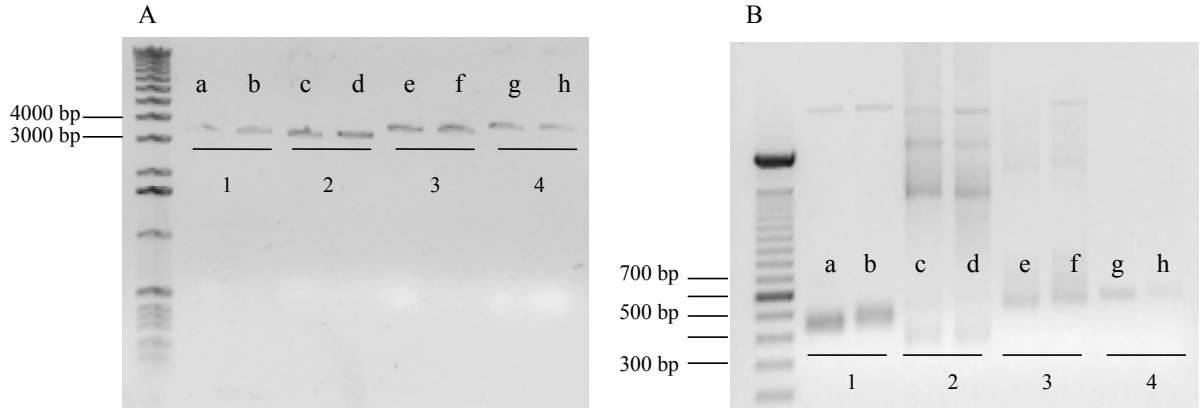


Figure 27. Synthesis of DIG-labeled riboprobes.
Numbers 1, 2, 3, 4 correspond to DEFB27, DEFB23, DEFB29 and ET-1 respectively. Letters a, c, e, g indicate sense probes and b, d, f, h, were assigned to antisense probes. (A) pGEM-T plasmids containing the inserts (total expected size 3000 bp-3500 bp) were linearized by using restriction endonucleases (ET-1 and DEFB27 by *Not* I; DEFB23 and DEFB29 by *Pst* I). Linear plasmids used to generate riboprobes were visualized on a 2% agarose gel using standard electrophoresis. (B) Efficiency of riboprobe synthesis. The amount of the corresponding riboprobes used during *in situ* hybridization was determined according to the results obtained by standard 2% agarose gel electrophoresis. Expected size of riboprobes was between 300 bp and 700 bp.

The presence of endothelin-1 and endothelin receptors in human testis was probed by PERI *et al.* For this reason this molecule was used as positive control. *In situ* hybridization was performed on slices from human caput epididymis. Tissues were kindly provided by A. Prützmann, St. Franziskus Hospital, Mönchengladbach, (Germany). The material was obtained from two different patients suffering from testicular tumors. Subsequently, tissues were separated into three different parts: lobuli testis, caput epididymis and cauda epididymis. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5 µm were spread on slides, deparaffined, rehydrated and buffered with PBS. *In situ* hybridization was then performed (see 2.5.4). After hybridization of human epididymal sections with digoxigenin-labeled riboprobes, a

secondary anti-digoxigenin alkaline phosphatase antibody was used to recognize the complex. The hybridization was subsequently detected by a colorimetric reaction with two substrates (BCIP and NBT) which form a reduction-oxidation system. In the particular cases of DEFB23 and DEFB27, the staining was restricted to the principal cells of the epididymal epithelium, but a signal was also present in the negative control (sense probe), (data not shown). To resolve this problem, several attempts to generate riboprobes using cDNA obtained (after the cloning process) from one single clone were carried out, but similar results were achieved. An evaluation of this investigation ended with no conclusive interpretation for these two β-defensins. However, high expression of DEFB29 in the epithelial cell layer lining the lumen of the proximal caput epididymis, was detected. A specific signal for DEFB29 antisense was located in dark color within epithelial cells of human caput epididymis (Figure 28, a). In contrast, no signal was detected in the negative control, performed with sense strand of DEFB29 riboprobe (Figure 28, b).

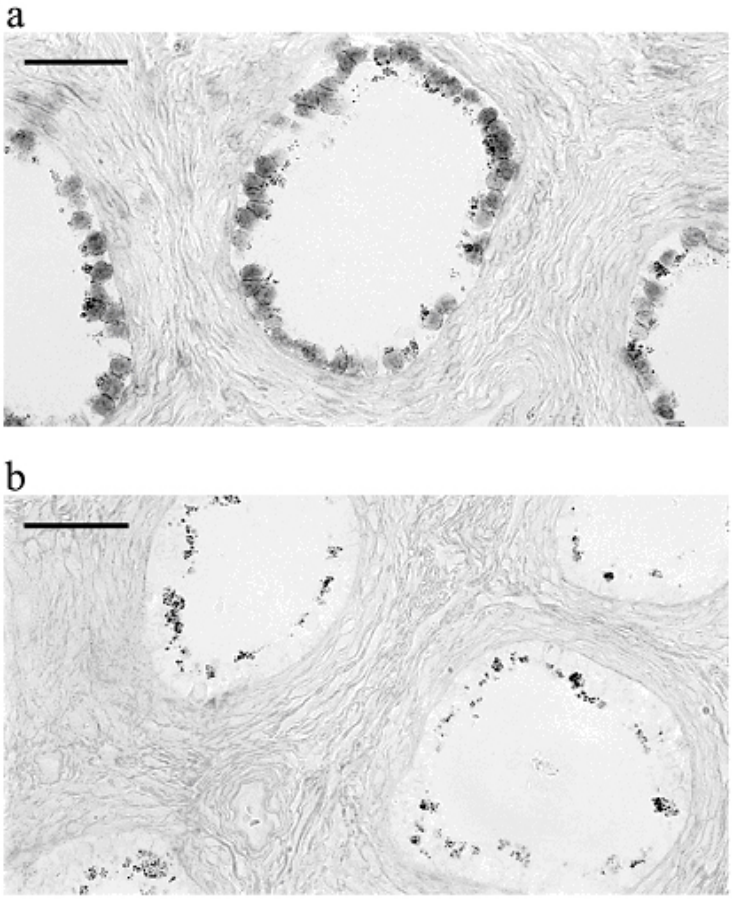


Figure 28. Non-radioactive *in situ* hybridization of DEFB29 in human caput epididymis.
In situ hybridization in human caput epididymis was performed for DEFB29. Transverse sections of human caput epididymis shows tubules with epithelial cells surrounding the lumen wherein the spermatozoa are located. The amount of sense (5 µl) and antisense (5 µl) probes applied was adjusted according to the amount of riboprobe determined by previous electrophoresis (Figure 27). Digoxigenin-labeled RNA probes of DEFB29 hybridized to tissue slices being recognized by a secondary anti-DIG alkaline phosphatase antibody and detected with NBT/BCIP. (a) Hybridization with the antisense probe. (b) Control with sense probe. Scale bars correspond to 50 µm. The experiments were reproduced three times.

Note that the apical granules of these cells were not labeled but appear dark. This is due to refractivity light in both sense and antisense experiments. Control experiments, such as omitting the probe or pretreatment with RNase A (100 µg/ml) (enzyme that destroys RNA), gave no signal above background (data not shown).

In order to clarify the nuclear or cytoplasmic location of the signal, the state of conservation of the cells after treatment as well as the cell distribution in the tissue, sections were treated with DAPI (fluorescent dye that stains the nuclear DNA). DAPI allowed determination that the signal was specifically located in the cytoplasm of the epithelial cell layer, lining the lumen of human epididymis. Although the nucleus occupied most of the cell, a thin line is visualized at the outer region (labeled by an arrow). Since the cytoplasm is around the nucleus, the staining was detected all over the cell. Observe that, in general, the cells present an optimal state of conservation after the *in situ* process. Non-epithelial cells are among tubules but did not present a signal. The staining was localized in epithelial cells confirming the cellular specificity for the detected DEFB29 mRNA. Experiments were repeated three times and similar results were obtained.

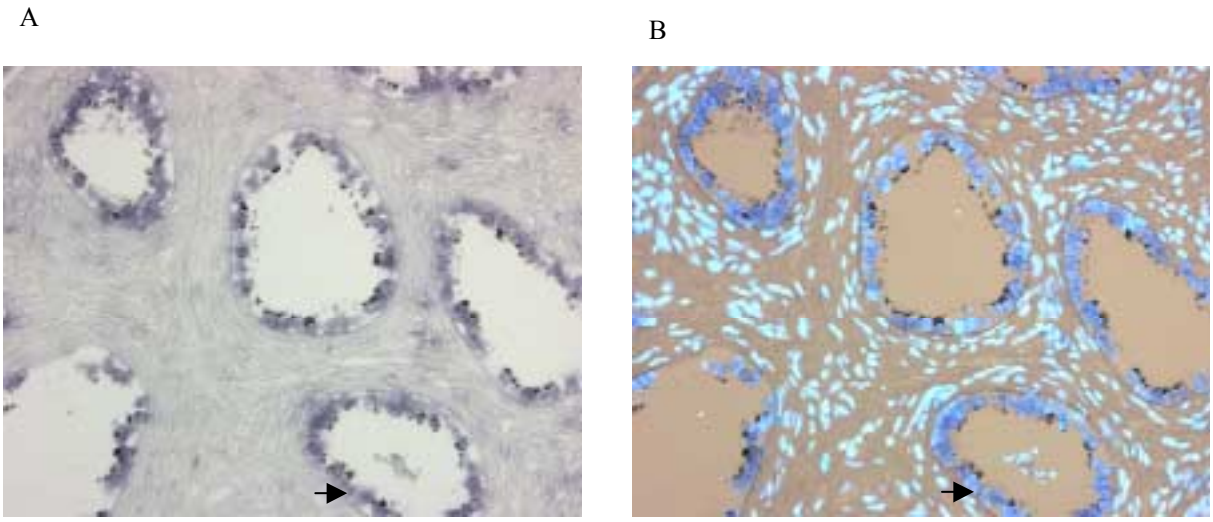


Figure 29. Location of DEFB29 mRNA in human epididymis.
Transversal sections (5 µm) of human caput epididymis show tubules with epithelial cells surrounding the lumen. (A) Non-radioactive *in situ* hybridization based on the use of DIG-labeled RNA probes of DEFB29. This probe hybridized to tissue slices being recognized by a secondary anti-DIG alkaline phosphatase antibody and detected with NBT/BCIP. Positive signal for DEFB29 mRNA is located in the cytoplasm of epithelial cells surrounding the epididymal lumen (B). On the same section, nuclear DNA was labeled using DAPI (1 mg/ml) and visualized by blue color (fluorescence detection). Non-epithelial cells are visualized among the tubules (Figure 29, B) but lack staining (Figure 29, A). Albeit the nucleus occupies most space inside the epithelial cell, the staining is located in the cytoplasm which envelopes the nucleus. In the panoramic overview, the cytoplasmic staining is also detected as a thin line in the extranuclear region (indicated by an arrow). Magnification × 20.

Additional experiments in lobuli testis and cauda epididymis were performed following the same protocol. In the case of cauda epididymis, only background and no specific signal was detected (data not shown). The glandular structure of the testis consists of numerous lobules. Within the

basement membrane cells are arranged in several concentric layers lining the central lumen of each individual lobule. The different germ cells may be seen in different stages of development. In lobules testis, the antisense signal was slightly above sense background staining. This situation impairs the analysis of the results and the identification of the different stained cells, raising no conclusive interpretation.

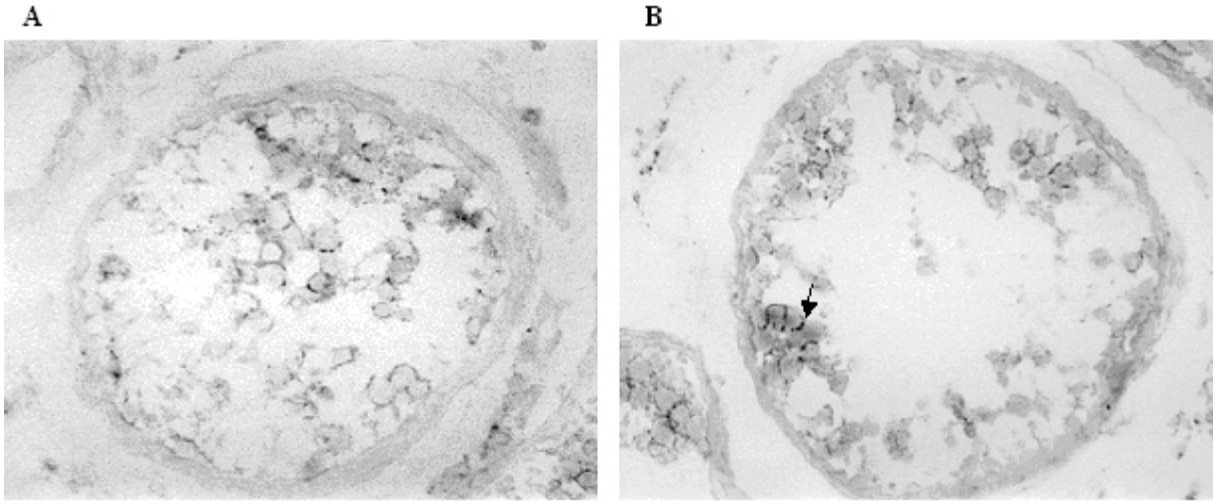


Figure 30. *In situ* hybridization of DEFB29 in human lobule testis.
Transversal sections (5 µm) of tubules of human lobule testis. DIG-labeled RNA probe of DEFB29 hybridized to tissue slices being recognized by a secondary anti-DIG alkaline phosphatase antibody and detected with NBT/BCI. Staining produced in human lobule testis, with sense (A) and with antisense DEFB29 riboprobe (B). Putative positive staining is indicated by an arrow. Magnification × 20.

3.10 Immunodetection of hBD4 in human epididymis

To detect and localize the presence of hBD4 in human epididymis, immunohistochemical studies were accomplished on paraffinized sections using monoclonal antibodies (see 2.9). The detection was performed by peroxidase staining. Antibodies for hBD23, hBD27 and hBD29 were not available, but the monoclonal antibodies against hBD4 were ready to use. The use of this antibody seemed to be reasonable since hBD4 presented a comparable expression pattern to the new genes in human epididymis.

3.10.1 Selectivity and sensitivity of anti-hBD4 monoclonal antibodies

The characterization of antibody selectivity requires demonstration that the antibody binds selectively to the protein that contains the immunogenic peptide.

Western blot is a suitable procedure to check the selectivity of an antibody which should recognize a single protein by visualization of a band at the appropriate molecular weight. Therefore a specific reaction of the antibody with the peptide or protein used for immunization (in comparison to related peptides) was performed by Western blot.

After immunization with a cyclic synthetic fragment of hBD4, (ELDRICGYGTARCRKKCRSQEYRIGRCPNTYACCLRK), several monoclonal antibodies were obtained. During the antibody production, a first screening searching the positive clones was performed by solid-phase ELISA. Antigen was applied to a microtiter-well containing hybridoma supernatants (monoclonal antibodies). The selection system is based on a colorimetric reaction and further measurement of the optical density by photometry. Using this technique the following positive clones (hybridomas) were detected:

L1310D1, L1310D2, L1310G5, L1310G6, L1310A12, L1310H10, and L11135a-D12.

For further immunohistochemical analysis, sensitivity and selectivity of the undiluted supernatant from the corresponding hybridomas were tested by Western blot. To study their sensitivity, the synthetic peptide was applied to the gel in four different concentrations (3 ng, 30 ng, 100 ng, 300 ng) (see 2.8.2). After blotting, all the described antibodies were able to detect 3 ng (data not shown).

To test their selectivity, 100 ng of synthetic peptide (hBD1, hBD2, hBD3 and hBD4) were confronted with each individual antibody (see 2.8.1). The results showed that the most specific antibody was L1310G6. This monoclonal antibody recognized specifically hBD4 and did not show crossreactivity with any other tested β-defensin (Table 16, line 3).

Table 16. Selectivity of anti-hBD4 monoclonal antibodies tested by Western blotting.

The symbols mean (-) lack of visible band. (+) Very faint band.+ Possible to appreciate a band. ++ Clear band. +++ Strong band.

Supernatant hybridoma	hBD1 (100 ng)	hBD2 (100 ng)	hBD3 (100 ng)	hBD4 (100 ng)
L1310D2	+	+	+	+++
L1310G5	+	(-)	++	+++
L1310G6	(-)	(-)	(-)	+++
L1310H10	(+)	(-)	+	+++
L1310A12	(-)	(-)	++	+++
L1310D1	(+)	(-)	(+)	+++
L11135aD12	+	(+)	+	+++

To test the crossreactivity for the most specific antibody (L1310G6), an additional analysis was performed. The selectivity was also studied by Western blot as previously described. The assay was carried out by addition of the antibody to a membrane containing:

-The synthetic hBD1, hBD2, hBD3 and hBD4 (all of them in cyclic form).

-The synthetic human β-defensins object of the present study: hBD27 (cyclic form), hBD23 (linear form), and hBD29 (linear form).

The L13-10G6 monoclonal antibody only recognized the synthetic hBD4 peptide in cyclic form (Figure 31, column 4).

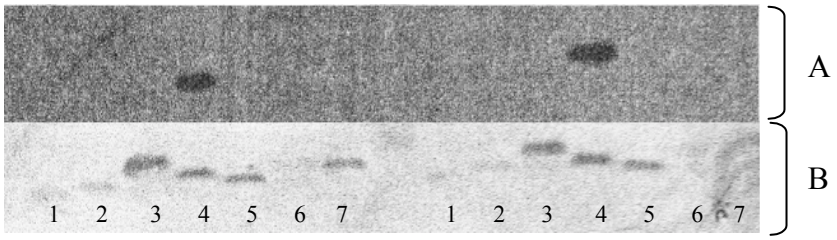


Figure 31. Selectivity of monoclonal antibody L13-10G6.
Seven peptides were included in the study: (1) 100 ng hBD1 cyclic form; (2) 100 ng hBD2 (cyclic form); (3) 100 ng hBD3 (cyclic form); (4) 100 ng hBD4 (cyclic form); (5) 100 ng hBD27 (cyclic form). (6) 100 ng hBD23 (linear form); (7) 100 ng hBD29 (linear form). (A) After SDS-PAGE, Western blot showed that the bands were detected only in column 4, where the cyclic form of hBD4 was applied. (B) Indian ink-stained membrane showed that seven peptides were loaded and blotted. The experiment was performed *in duplica*.

3.10.2 Immunohistochemistry for hBD4

Immunohistochemistry is a powerful method for the identification of proteins in cells and tissues. This method depends on the selectivity and sensitivity of the antibody binding to the epitope of the protein used as immunogen.

To detect and localize the presence of hBD4 in human epididymis, immunohistochemical studies were performed on paraffinized sections, using the anti-hBD4 monoclonal antibody (L1310G6). The previously mentioned, anti-hBD4 monoclonal antibodies were also tested during this study. The antibody that presented the clearest signal by immunohistochemistry (L1310G6) was also the most specific antibody after Western blot evaluation.

Immunostaining was performed using the undiluted supernatant of hybridoma termed L1310G6 containing an anti-hBD4 monoclonal antibody. The staining appeared in at least three locations:

1-In the supranuclear Golgi region of basal epithelial cells.

2-In cytoplasmic accumulations close to the lumen. According to other reports these spots might be cytoplasmic vesicles (GLICK and MALHOTRA, 1998).

3-Around the sperm.

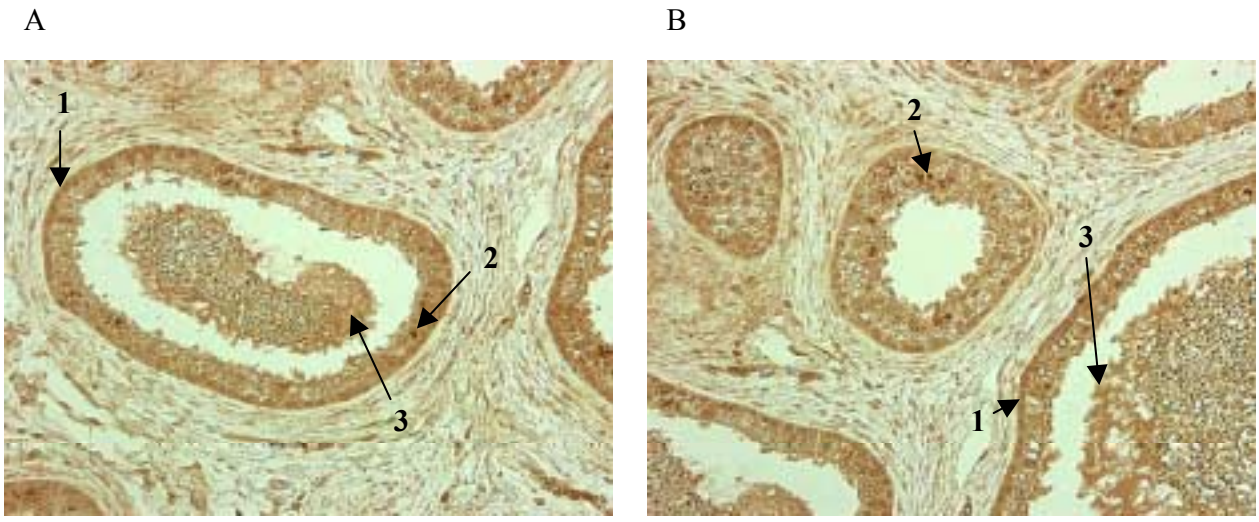


Figure 32. Immunodetection of hBD4 in proximal caput segment of human epididymis.
Immunohistochemistry of the caput region of human epididymis. The figures show tubules with the corresponding epithelial cells surrounding the lumen wherein the spermatozoa are located. Interstitial tissue envelops the tubules. (A and B) Binding of the specific undiluted supernatant containing the anti-hBD4 monoclonal antibody (L1310G6) was visualized by a peroxidase reaction, resulting in brown deposits located in three different positions: (1) Supranuclear Golgi region in basal epithelial cells. (2) Cytoplasmic accumulations. (3) In seminal plasma surrounding the sperm located in the center of the tubule. Magnification, × 20.

Dilutions of the antibodies used are usually recommended as controls for testing the selectivity of the staining in tissues. A dilution of 1/5 of the described monoclonal antibody L1310G6 against hBD4 was included in this study, showing a weaker positive signal.

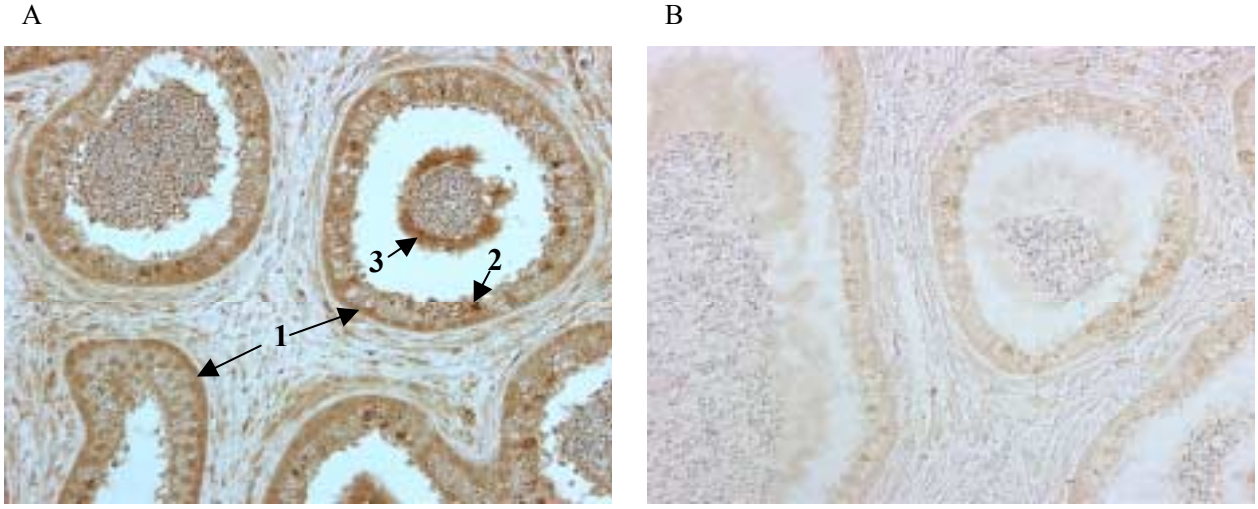


Figure 33. Immunodetection of hBD4 in distal caput segment of human epididymis.
Immunohistochemistry of the caput region of human epididymis. The figures show tubules with the corresponding epithelial cells surrounding the lumen wherein the spermatozoa are located. Interstitial tissue envelops the tubules. (A) Binding of the specific undiluted supernatant containing the anti-hBD4 monoclonal antibody (L1310G6) was visualized by a peroxidase reaction, resulting in brown deposits located in three different positions: (1) Supranuclear Golgi region in basal epithelial cells. (2) Cytoplasmic accumulations. (3) In seminal plasma surrounding the sperm located in the center of the tubule (B) Staining detected by a 1/5 dilution of L1310G6 monoclonal antibody. Magnification, × 20. A bright light filter was used for better comparison.

The proximal region of caput epididymis is closer to rete testis. The distal caput epididymis is closer to corpus epididymis. Sections from proximal caput epididymis (Figure 32) presented less

stained cytoplasmic spots in comparison to distal caput epididymis (Figure 33, A). The locations in basal epithelial cells and around the sperm cells were also present in early sections. Controls for the selectivity of an antibody are important for the correct interpretation of its localization in cells and tissues. One control for selectivity is termed absorption or preabsorption. For this control, the antibody is mixed with the protein or peptide used to generate the antibody, with the objective to eliminate or reduce the binding of the antibody to the protein in the tissue. Preincubation of the synthetic hBD4 (10 µg/ml, cyclic form) with the supernatant of hybridoma containing the anti-hBD4 monoclonal antibody (L1310G6) was carried out. The antibody was raised against the same hBD4 cyclic fragment used in preabsorption experiments. A specific binding of hBD4-L1310G6 (Ag-Ab) was expected prior to the incubation on the sections. That would result in less available antibody for recognizing hBD4 in tissues and therefore in a weaker signal. Strikingly, a darker staining was detected, but also with more non-specific binding (Figure 34, A). However, the same previously described positive locations identified by L1310G6 were observed. Note that the signal in figure 34, A, is more intense in comparison to figure 32. The signal was clearly amplified, especially for the cytoplasmic spots. The experiments were repeated four times and similar results were obtained. In order to obtain more information about this surprising result obtained by preabsorption, a similar experiment was scheduled. A preabsorption experiment by incubating the synthetic peptide hBD1 (10 µg/ml, cyclic form) with the anti-hBD4 monoclonal antibody (L1310G6) was carried out.

A B

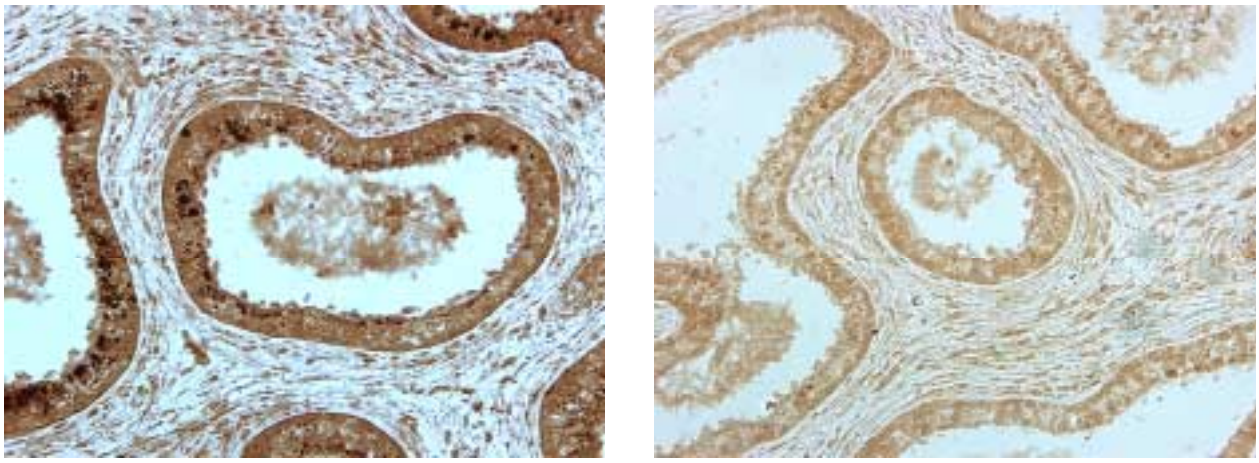


Figure 34. Preabsorption of monoclonal anti-hBD4 antibody (L1310G6) with synthetic hBD4 (cyclic form) and hBD1 (cyclic form).
Immunohistochemistry of transversal sections of human caput epididymis. The figures show tubules with the corresponding epithelial cells surrounding the lumen wherein the spermatozoa are located. Interstitial tissue envelops the tubules. Binding of antibody is visualized by a peroxidase reaction, resulting in brown deposits. The preabsorption of antibody with antigen was performed prior to adding on the human epididymal sections. Immunostaining obtained by immunohistochemistry occurred after the preabsorption of the monoclonal antibody L1310G6 with hBD4 (cyclic form) (A) or hBD1 (cyclic form) (B). Magnification, × 20.

According to the results obtained by Western blot, L1310G6 monoclonal antibody was specific for hBD4 and did not detect hBD1. As expected, the results reflected that the staining appeared in the same locations than those incubated exclusively with L13 10G6 and with a comparable intensity (Figure 34, B).

Sections of human epididymis with cells containing a certain protein with demonstrated location within the tissue, could be used to optimize the method. Endothelin-1 (ET-1) is a peptide involved in eliciting smooth muscle cell with contractile activity in different tissues and organs, including human epididymis (PERI *et al.*, 1997).

A specific immunostaining for ET-1 was detected in the epithelial location within the epididymal tract. For this reason this molecule was used as positive control. The present results are consistent with those previously described within the epithelial cells of the human epididymis duct wall (PERI *et al.*, 1997). However, this work also showed a signal in apical spots (marked by an arrow in figure 35).

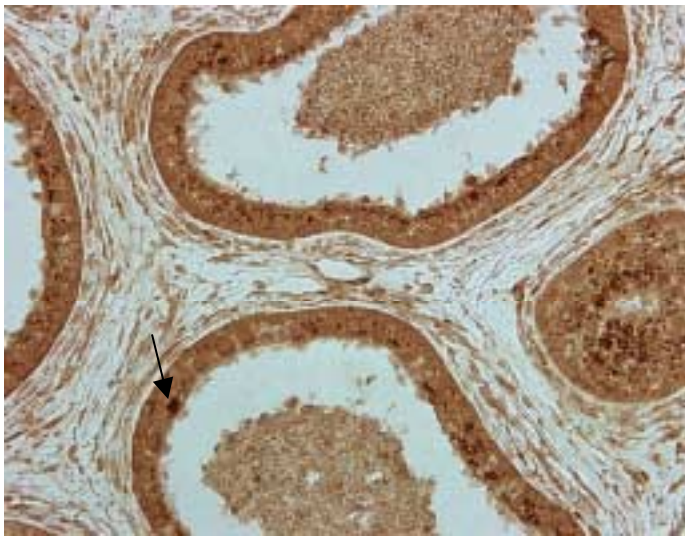


Figure 35. Immunolocalization of ET-1 (positive control) in human epididymis.
Immunohistochemistry of transverse sections of human caput epididymis. The figures show tubules with the corresponding epithelial cells surrounding the lumen wherein the spermatozoa are located. Interstitial tissue envelops the tubules. Binding of antibody is visualized by a peroxidase reaction, resulting in brown deposits. Positive immunostaining for ET-1 was detected in epithelial cells and in their apical spots (labeled by an arrow).

The selectivity of the immunohistochemical procedure was validated by negative controls which ensured that the labeling method accurately identified the antibody bound to the tissue. Because of the variety of background and non-specific labeling which are inherent in any immunohistochemical analysis of cells and tissues, some negative controls were included.

The negative control slide consisted of a section which was treated identically to the rest of slides but the primary antibody was omitted. The negative control slide was subjected to the same epitope retrieval procedures (protease, microwave, etc.) as the primary antibody. The

reason was to avoid that the epitope-preparation steps can significantly intensify endogenous artifacts. The following negative controls were included in the study.

1-The intact culture medium Optimem was used as negative control, replacing the primary antibody. This medium was applied, because Optimem was the tissue culture medium used to culture the hybridoma whose supernatant contained the monoclonal antibody L1310G6. The results showed that no specific signal could be detected using culture medium instead of primary antibody.

2-An isotype IgG γ-1 from mouse was an additional negative control used to estimate the amount of non-specific binding of mouse monoclonal antibodies to the human cell-surface and antigens in human epididymis. The IgG γ-1 mouse antibody is the same type of immunoglobulin (isotype), keeping the same three-dimensional structure of L1310G6, but it lacks specific regions of antigen recognition. For this reason, the isotype IgG γ-1 should not recognize hBD4 in the tissue. The use of this antibody resulted in no signal above background.

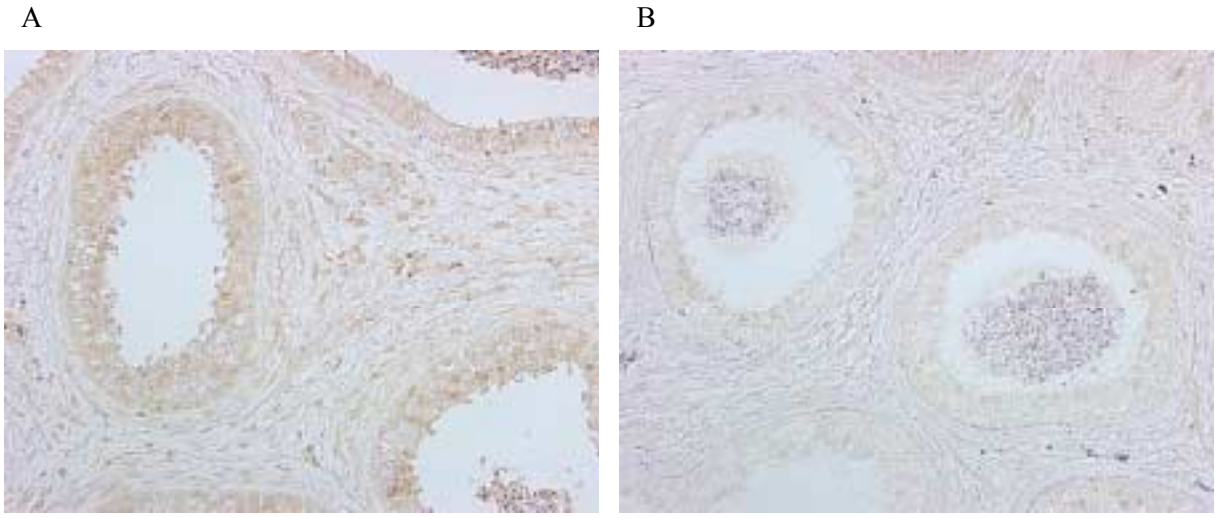


Figure 36. Negative controls used in immunodetection of hBD4.
Immunohistochemistry of transversal sections of human caput epididymis. The pictures show tubules with the corresponding epithelial cells surrounding the lumen wherein the spermatozoa are located. Interstitial tissue envelops the tubules. The detection of the bound antibody is based on the visualization of brown deposits due to a peroxidase reaction. (A) Negative control Optimem culture medium replaced the primary antibody, resulting in loss of labeling. (B) The specific primary antibody was replaced by non-immune Isotype IgG γ-1 mouse antibody and gave no staining above background.

Hybridoma supernatants (monoclonal antibodies) were screened by enzyme-linked immunosorbent assay (ELISA) to detect positive clones, for example L1310G6 and L1310D1. Since every antibody presents differences in the variable region, the results obtained with every clone can differ substantially from one another. In fact, and in contrast to the positive results shown by using the positive clone L1310G6, no specific signal was detected by anti-hBD4 monoclonal antibody L1310D1.

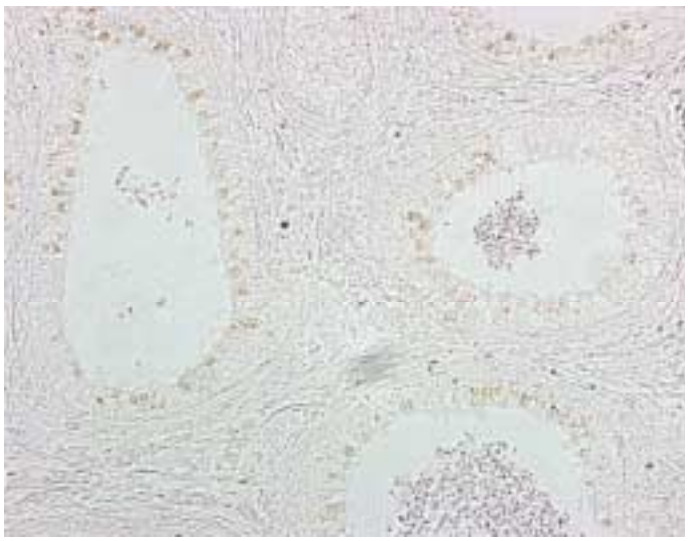


Figure 37. Immunolocalization of hBD4 in human epididymis by monoclonal antibody L1310D1.
Immunohistochemistry of transversal sections of human caput epididymis. The pictures show tubules with the corresponding epithelial cells surrounding the lumen wherein the spermatozoa are located. Interstitial tissue envelops the tubules. The detection of the bound antibody is based on the visualization of brown deposits due to a peroxidase reaction. After the test system ELISA, another monoclonal antibody (L1310D1) was considered as a positive hybridoma. This clone was obtained after immunization with the same antigen (hBD4 cyclic form). Sections incubated with anti-hBD4 monoclonal antibody L1310D1 showed no staining.

3.11 Evaluation of antimicrobial activity for hBD23, hBD27, and hBD29

3.11.1 Radial diffusion assay

Defensins have been defined so far as small cationic peptides that are crucial components of innate immunity. The specific mechanism of antimicrobial activity involves permeabilization of bacterial membranes (see 1.2).

Many studies have demonstrated that human β-defensins, e.g. hBD1 (VALORE *et al.,* 1998), hBD2 (TOMITA *et al.,* 2000), hBD3 (GARCIA *et al.*, 2001-A), hBD4 (GARCIA *et al.*, 2001-B) are antimicrobial agents. Antimicrobial activity of the new β-defensins was tested by radial diffusion assay. The commonly used radial diffusion assay method is relatively simple to perform and is a widely employed method in the laboratory since it provides semiquantitative or qualitative data about the susceptibility of a given organism to a given agent. Experiments were repeated twice and the results of this study showed that hBD27 (cyclic form) did not exert antimicrobial activity against any of the tested bacteria, even at twice the concentration of the positive control. In contrast, hBD23 and hBD29 (10 µg, linear form) presented antimicrobial activity against *Klebsiella pneumoniae* DSM681, *Pseudomonas aeroginosa* 1128, *Escherichia coli* 1103, and *Escherichia coli* 96440.

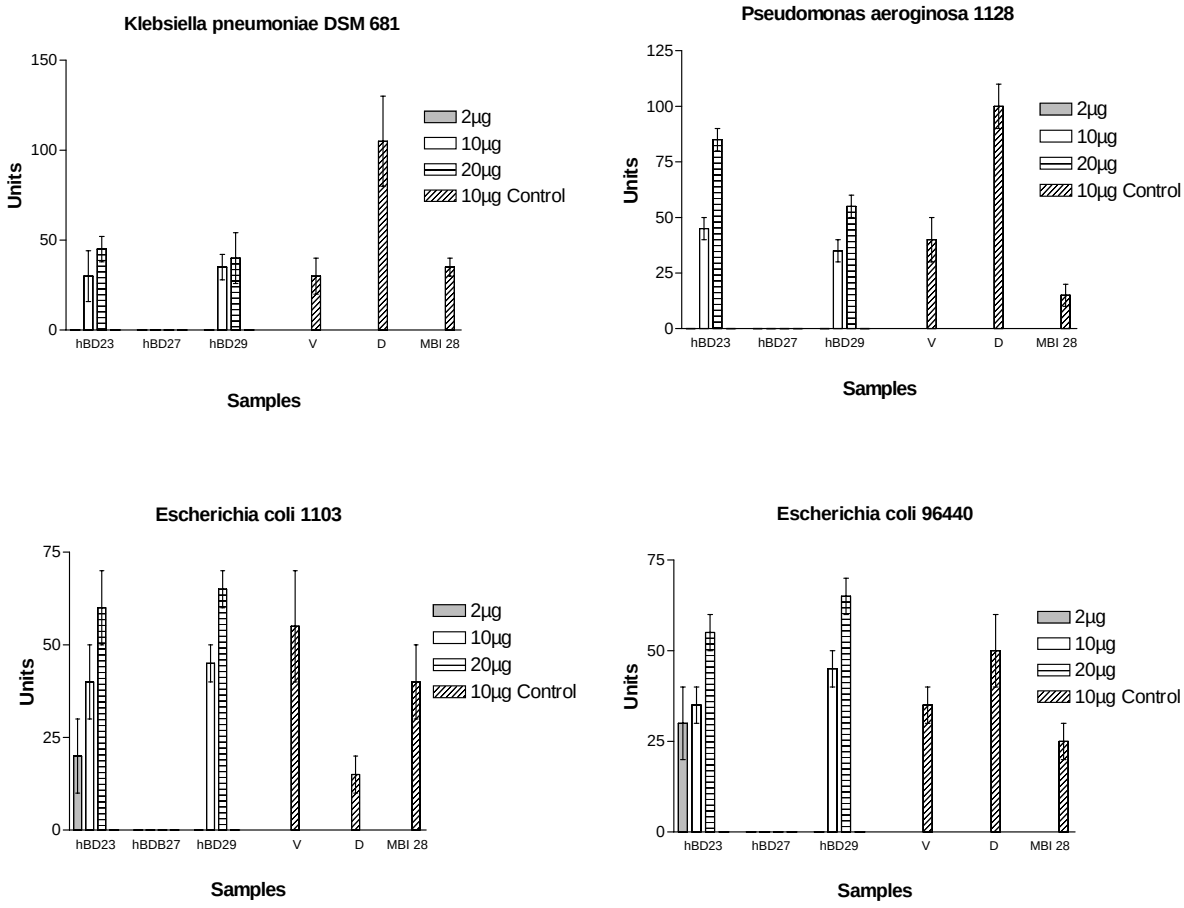


Figure 38. Antimicrobial activity of the new β-defensins against four different bacterial strains.
The antimicrobial activity of hBD23, hBD27, hBD29 was evaluated by radial diffusion assay. The experiments were fulfilled using three different concentrations of peptide (2 µg, 10 µg, 20 µg) and including three positive controls MBI28 (10 µg), vancomycin (V) (10 µg) and doxycycline (D) (10 µg). The bacteria tested were *Klebsiella pneumoniae* DSM681, *Pseudomonas aeroginosa* 1128, *Escherichia coli* 1103, and *Escherichia coli* 96440.

On the other hand, none of the new β-defensins presented significant antimicrobial activity against *Staphylococcus aureus* ATCC 25923 and *Streptococcus pneumoniae* DSM 11865.

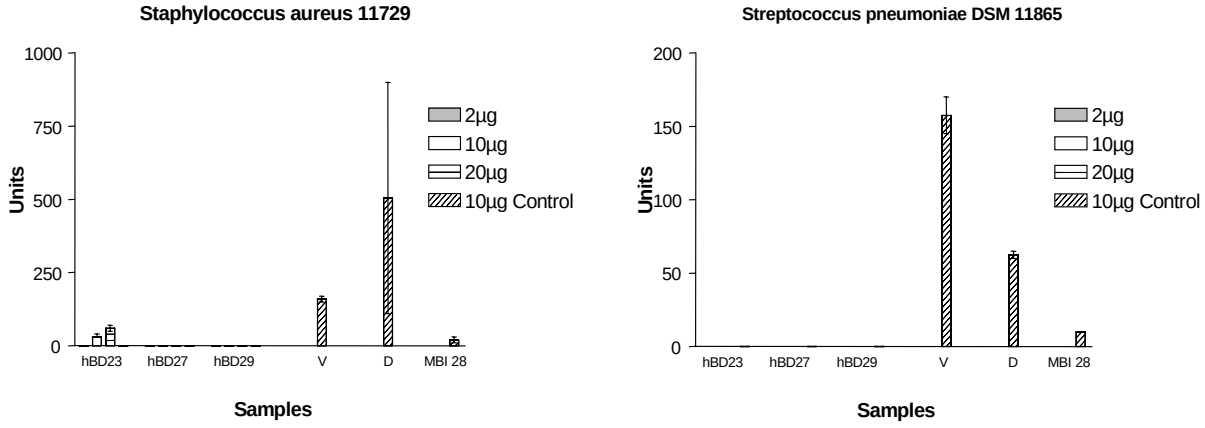


Figure 39. Antimicrobial activity of the new β-defensins against two different bacterial strains.
The antimicrobial activity of hBD23, hBD27, hBD29 was evaluated by radial diffusion assay. The experiments were fulfilled using three different concentration of peptide (2 µg, 10 µg, 20 µg) and including three positive controls MBI28 (10 µg), vancomycin (V) (10 µg) and doxycycline (D) (10 µg). The bacteria tested were *Staphylococcus aureus* ATCC 25923 and *Streptococcus pneumoniae* DSM 11865.

3.11.2 Determination of the minimal inhibitory concentration

Minimal Inhibitory Concentration (MIC) is defined as the lowest concentration of drug that inhibits more than 99% of the bacterial population. The determination of the minimum inhibitory concentration (MIC) is apart from the radial diffusion assay, a second method for the determination of the antimicrobial activity of a certain material. The MIC value represents the reference for the antimicrobial effectiveness of a substance, being usually indicated in the literature of an antimicrobial agent. For a group of antibiotics such as quinolones, or aminoglycosides, a ratio of concentration: MIC 10 is required. This implies that a dose regimen should be chosen which results in a serum or tissue concentration of at least 10 times the MIC. The technique contributes information about the suitable concentration to be used. Failure to achieve this concentration at the site of infection will lead to clinical and bacteriological failure, and is likely to induce resistance to the entire class of antibiotic. The technique offers important information about the antimicrobial potency of a substance. Several strains of bacteria were included in the study for a better and complete overview of their antimicrobial potency.

Table 17. Minimal inhibitory concentration for hBD23.

Bacteria strain	Gram staining	MIC_{MHB} [µg/ml]
Staphylococcus carnosus TM300	+	18,8
Staphylococcus aureus ATCC25923	+	75
Streptococcus pneumoniae DSM11865	+	75
Escherichia coli DSM 1103	-	37,5
Klebsiella pneumoniae DSM681	-	75
Pseudomonas aeruginosa DSM 1128	-	150

Table 18. Minimal inhibitory concentration for hBD27.

Bacteria strain	Gram staining	MIC_{MHB} [µg/ml]
Staphylococcus aureus ATCC25923	+	>300
Streptococcus pneumoniae DSM11865	+	>300
Escherichia coli DSM 1103	-	>300
Klebsiella pneumoniae DSM681	-	>300
Pseudomonas aeruginosa DSM 1128	-	>300

Table 19. Minimal inhibitory concentration for hBD29.

Bacteria strain	Gram staining	MIC_{MHB} [µg/ml]
Staphylococcus carnosus TM300	+	37,5
Staphylococcus aureus ATCC25923	+	>300
Streptococcus pneumoniae DSM11865	+	>300
Escherichia coli DSM 1103	-	>300
Klebsiella pneumoniae DSM681	-	>300
Pseudomonas aeruginosa DSM 1128	-	>300

The order of magnitude of 1-8 µg/ml is considered as an acceptable MIC value for clinically relevant cationic peptide, described by HANCOCK (1997).

The present results show that, with the described techniques and in the range of the used concentrations, hBD27 did not present any antimicrobial activity against the germs tested.

3.11.3 Hemolytic activity

Due to the fact that β-defensins base their antimicrobial activity in the interaction with the membranes of bacteria, it is important to know whether these peptides can upset the membrane of human cells.

The hemolytic activity of hBD23 and hBD27 was investigated in a hemolytic assay using human erythrocytes in order to know the disruption or damage that these substances can cause to human erythrocytes. A sensitive assay described by HELMERHORST *et al*., 1999 was used to study their hemolytic effect (see 2.11).

The percentage of cell lysis produced by hBD27 was determined to be independent of the concentration within the range of 10 µg/ml to 100 µg/ml. However, a slight increase was observed with a concentration of 500 µg/ml. No significant hemolytic activity was noticed for hBD27 in the range of the concentrations tested.

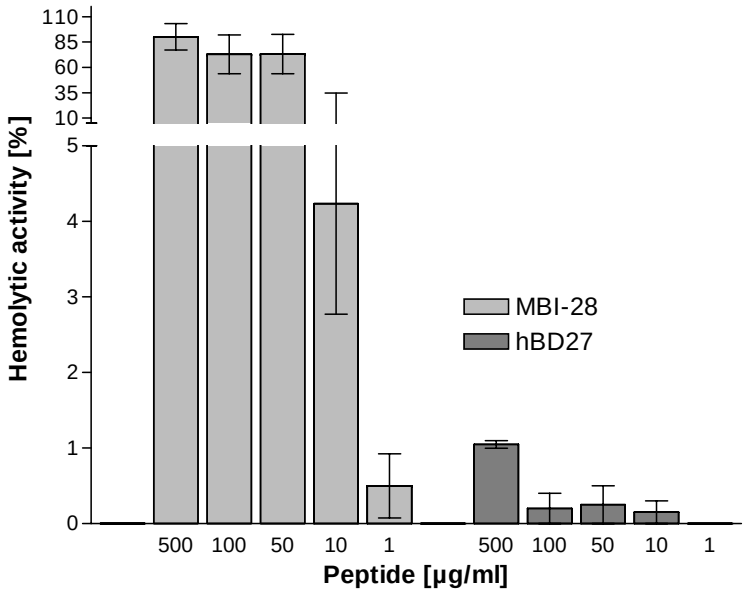


Figure 40. Hemolytic activity of hBD27.
The hemolytic activity of hBD27 was analyzed by the measurement of free hemoglobin released by erythrocytes after disruption. Free hemoglobin was determined photometrically in a microplate reader. Synthetic hBD27 (cyclic form) was applied in different concentrations (1-10-50-100-500 µg/ml). MBI28 was used as positive control used under the same experimental conditions. Test medium (1/10 TSB, 287 mM glucose, pH 7.4) was included as negative control with complete absence of hemolytic activity. Experiments were repeated three times and similar results were obtained.

The percentage of hemolytic activity for hBD23 depends on the concentration of peptide. The application of 500 µg/ml of synthetic hBD23 (linear form) resulted in 80% of cell lysis.

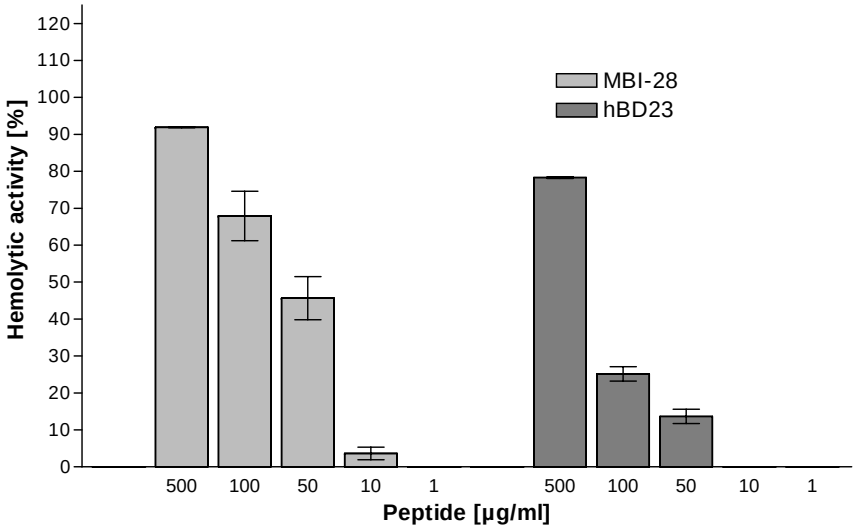


Figure 41. Hemolytic activity of hBD23.
The hemolytic activity of hBD23 was analyzed by the measurement of free hemoglobin released by erythrocytes after disruption. Free hemoglobin was determined photometrically in a microplate reader. Synthetic hBD27 (cyclic form) was applied in different concentrations (1-10-50-100-500µg/ml). MBI28 was used as positive control used under the same experimental conditions. Test medium (1/10 TSB, 287 mM glucose, pH = 7.4) was included as negative control with complete absence of hemolytic activity. Experiments were repeated three times and similar results were obtained.

4. Discussion

The aim of this study was to identify and characterize novel human β-defensins, a family of cationic peptides considered as antimicrobial agents. At the start, the investigation was focused on the search for new genes containing the characteristic six cysteine motif of the β-defensin family by *in silico* screening of genomic sequences. After discovering three novel genes (DEFB23, DEFB27, and DEFB29), description, expression and regulation of the genes as well as the location and biological role of the putative peptides in the human organism were scheduled and performed. The interpretation of the results obtained is presented in the following chapters.

4.1 Features of the new genes and their corresponding amino acid sequences

Genome sequencing efforts are revolutionizing biomedical science. The relevance of functional genomics in modern science has become more important since the Human Genome Project (HUGO) was completed. Functional genomics involves high throughput techniques and bioinformatical tools that facilitate the prediction of the biological function of genes and proteins (or parts of them) based on structural data.

Using this scientific strategy, the present work reports the discovery of three new human β-defensins, named DEFB23, DEFB27, and DEFB29. The novel genes were identified by the analysis of genomic sequences (see 2.13 and 3.1). The new genes are mapping on chromosome 20 in two different clusters. DEFB27 and DEFB29 are clustered at the telomere (accession number of the contig: AL360078) and DEFB23 at the centromere (accession number of the contig: AL121751) of the chromosome. This location differs from the chromosomal location of human defensins described so far, which are encoded in the chromosomal region 8p23. The genes encoding the leukocyte α-defensins (HNP) are comprised of three exons (KAISER and DIAMOND, 2000). The α-defensins HD5 and HD6 consist of two exons, which are equivalent to exons two and three of the leukocyte α-defensins (QUAYLE *et al*., 1998). The new β-defensins (DEFB23, DEFB27, and DEFB29) exhibit the same genomic organization as other β-defensins previously described (DEFB1, DEFB2, DEFB3, DEFB4, and DEFB6), with two exons flanking an intron that differs in length for every new β-defensin. However, DEFB5 consists of three exons like the above-mentioned leukocyte α-defensins. The gene structure of the β-defensins suggests a second subdivision of the family (KAISER and DIAMOND, 2000). One subfamily, which includes DEFB2, contains a relatively small (2 kb) intron. The other subfamily includes

DEFB1 with a large intron (6972 bp). Interestingly, DEFB27 and DEFB29 show a much shorter intron (1000-2000 bp) in comparison to hBD23 (6000-7000 bp) (Table 14) which is encoded in a different chromosomal location (Figure 17). According to the classification proposed by Kaiser and Diamond, DEFB27 and DEFB29 might be included in the first subfamily, whereas DEFB23 would be in the second.

A comparison to the genomic sequences reveals that the novel cDNAs correspond to transcripts from two exons, wherein the signal peptide is encoded by the first exon and the cysteine-containing core structure by the second (Figure 13, Figure 14, and Figure 15).

HE2 was characterized as an epididymis-specific secretory protein, originally identified during a screening for epididymis-specific transcripts (OSTERHOFF *et al*., 1994). HE2 undergoes different splicing events leading to nine variants. Three variants (HE2C, HE2ß1, and HE2E) share the characteristic β-defensin cysteine pattern (VAN HORSTEN *et al*., 2002). The HE2ß1 variant contains three exons and is located on chromosome 8, between DEFB3 and DEFB4 (Figure 6). Thus, it is reasonable to propose that chromosomal location does not necessarily predict genetic structures.

The corresponding exon-intron boundary regions of DEFB23, DEFB27, and DEFB29 exhibit conserved splicing sites (Figure 16). Therefore, the RNA of these defensins might be processed by a similar splicing machinary.

Fragments of DNA encoding the classical six cysteine pattern of β-defensins, termed DEFB16 and DEFB22, were found in a centromeric contig of chromosome 20 (Figure 17). These sequences were considered as pseudogenes since neither ESTs searched *in silico* in public domain servers, nor amplification products following RACE-PCR were detected. These pseudogenes might not be expressed because during evolution they might have lost their physiological role in the organism.

The structural comparison of amino acid sequences is based on the results obtained *in silico* with the signal peptide prediction program (SignalP) and the alignment of the amino acid sequences deduced from the corresponding cDNAs. The amino acid sequences of hBD23, hBD27, and hBD29 were compared to the sequences of hBD1, hBD2, hBD3, hBD4, hBD5, hBD6, the mentioned pseudogenes, as well as two splicing variants of HE2 (HE2C and HE2E) (Figure 18). It is interesting to note that a glutamic acid residue, in fourth position carboxy-terminally located after the third cysteine, is conserved in most of them (including the new β-defensins). This glutamic acid is also conserved in the α-defensin family (Figure 2). Few conserved residues have been indicated, except the cysteine motif, between the α-defensin and β-defensin family. Nevertheless, it has been questioned whether the two families have descended from a single

ancestral gene. This common feature would contribute to supporting the evolutionary continuity between both families.

According to the results obtained by bioinformatical search using the SignalP server, the novel hBD23, hBD27, and hBD29 peptides contain secretory signal peptides indicating that they are amino-terminally processed and secreted from the cells.

In contrast to the primary structure of published β-defensin peptides (whose genes are mapping on chromosome 8p23) and hBD23 (encoded in centromeric region of chromosome 20q11.1), the new β-defensins hBD27 and hBD29 (chromosome 20p13) exhibit long carboxy terminal extensions following the β-defensin-specific cysteine core (Figure 18). These carboxy-terminal prolongations differ significantly in amino acid composition and do not exhibit homology with other sequences. For α-defensins, amino-terminal cationic prosequences are known to serve as regulatory domains (WILSON *et al.*, 1999), but the contribution of carboxy-terminal elongations to β-defensin function remains to be analyzed. Therefore, the isolation of the native peptides corresponding to the new β-defensins genes would be an important achievement.

4.2 Tissue distribution and regulation of the novel β-defensins

Examination of the expression pattern and tissue distribution of the new β-defensins was performed by using real-time quantitative PCR (*TaqMan*), resulting in the quantification of their relative transcription levels in 28 human tissues. Interestingly, only very few tissues show expression of the novel β-defensin genes. The three genes described here show the highest expression in the male reproductive organ (human testis and epididymis) and a considerable level of transcripts in skeletal muscle (Figure 22). The expression of DEFB29 is confined to these two organs (Figure 21). Beside these two organs, DEFB23 is expressed at lower levels in lung and trachea, tissues that also express other defensins (ZHAO *et al.*, 1996; BALS *et al.*, 1998) (Figure 19). Aside from the male reproductive organ and skeletal muscle, DEFB27 is expressed in kidney (Figure 20). Renal expression for DEFB1 (VALORE *et al.*, 1998) and the murine homologue mBD-1 (MORRISON *et al.*, 2002) was proposed to provide protection against urinary tract infection.

The expression of DEFB27 is more widespread over different tissues in comparison to the other two human β-defensins whose expression is detected in very few tissues and therefore with a higher spatial selectivity for developing their still unknown physiological functions. The expression analysis shows that none of the newly described β-defensins are constitutively

expressed in skin, as would be expected for peptides primarily involved in innate immunity (Figure 22).

The expression in the male reproductive organ is consistent with the results obtained in other investigations. For instance, recent publications show that hBD1 is expressed in testicular tissue and is present in ejaculated spermatozoa and the seminal plasma (COM *et al.*, 2003). Another investigation shows that the rat β-defensin 1 (RBD-1) is expressed in caput epididymis (PALLADINO *et al.*, 2003). Recent studies indicate that DEFB4 is highly expressed in the male reproductive tract (GARCIA *et al.*, 2001-B). The high level of DEFB4 expression in testicular tissue is attributed to the fact that during the extraction of RNA from testis, epididymal tissue was possibly included.

The mentioned studies, together with the recently described DEFB5 and DEFB6, which are specifically expressed in human epididymis (YAMAGUCHI *et al.*, 2002), reinforced the necessity of a more detailed study of expression within human epididymis. The present work achieves this demand and provides evidence that the novel members of the β-defensin family described here, show a distinct expression in the male genital tract, especially in functionally different segments of human epididymis. A global quantitative comparison of the expression profiles in the genital tract for the three new genes was performed. Along the organ, the expression of DEFB4 and DEFB23 exhibits a different tendency in comparison to DEFB27 and DEFB29 (Figure 26). The absolute abundance of mRNA in epididymis for DEFB4 and DEFB23 is lower than for DEFB27 and DEFB29. These results might be attributed to their different chromosomal location. On the other hand, this epididymal expression does not seem to be directly associated with their antimicrobial function, since DEFB27 was highly expressed in epididymis but did not exhibit antimicrobial activity (Figure 38 and Figure 39). Thus, some β-defensins might be mere antimicrobial peptides but this analysis suggests other putative roles for some of the new members of this peptide family.

In general, the epididymis displays a highly region- and cell-specific pattern of gene expression (RODRÍGUEZ *et al.*, 2001; ORGEBIN-CRIST *et al.*, 1995). Sperm maturation is accompanied by distinct temporal and spatial gene expression along the epididymis (KRULL *et al.*, 1993; PERA *et al.*, 1994). Region-specific gene expression in the epididymis implies regionalization of regulatory mechanisms (KIRCHHOFF *et al.*, 1997; KIRCHHOFF *et al.*, 1999). This specific regionalized expression, as was previously proposed for other β-defensin related peptides (FROHLICH *et al.*, 2001; LI *et al.*, 2001), may contribute to generate the optimal conditions and the necessary microenvironment for sperm maturation, storage and progression. Only recently, theories have been developed about how and when, during ontogenesis, this pattern formation

takes place and what its significance might be. The functional significance of the regional expression and the post-testicular modifications of the sperm surface occurring within the epididymis is still unclear. The order with which the sperm surface comes into contact with epididymal secretory proteins may be important for the resulting molecular and physiological effects. In fact, Chimpanzee spermatozoa obtained from the caput epididymis are immotile and unable to bind or to penetrate the ovum, but acquire these abilities when they reach the caudal region (GOULD and YOUNG, 1990).

The constitutive expression in skeletal muscle of all the newly described β-defensins is also known from the murine defensin mBD-6 (YAMAGUCHI e*t al., 2001*). This location is rather surprising, since this organ is not a frequent target for bacterial pathogens. A specific fluorescence for actin, alpha-actinin, and tropomyosin was detected in the principal cells of the bovine epididymal epithelium (YAGI *et al.*, 1992). These molecules are also structural components of the skeletal muscle. Similarities in the location for contractile proteins and defensins suggest further investigations, most importantly immunohistochemical assays to elucidate the exact location of the new defensins within this tissue. Such an investigation will help to elucidate the whys and wherefores of skeletal muscle expression.

It is known that certain peptides are produced in large quantities at sites of infection or inflammation, and their expression can be induced by bacterial products such as endotoxic lipopolysaccharide (LPS) and proinflammatory cytokines such as tumor necrosis factor α (TNF α). Concerning the regulation of the human β-defensin family, DEFB1 has been considered to be constitutively expressed in kidney and in epithelia of the female urogenital tract (VALORE *et al*., 1998). However, in blood cells, the expression of this gene was enhanced after stimulation by lipopolysaccharide or bacterial cells (FANG *et al*., 2003). Other authors have assumed that, due to the low levels of DEFB1 expression, regulation of DEFB1 is not conclusive (KING *et al*., 2002). DEFB2 can be induced in various epithelial tissues by inflammatory cytokines (BECKER *et al.*, 2000), or by bacteria and fungi (HARDER *et al*., 1999). On the other hand, DEFB3 and DEFB4 reside in epithelial but also in non-epithelial tissues and they can be induced by different inflammatory factors. In fact, DEFB3 is induced in certain cell lines by TNF α and bacteria (HARDER *et al*., 2001), as well as by γ-interferon (GARCIA *et al*., 2001-A). Recent results show that the expression for DEFB4 is inducible in lung epithelial cells by addition of PMA (GARCIA *et al*., 2001-B). Further evidence regarding the varying inducibility for every defensin is the inhibition of mRNA expression for DEFB3, but not for DEFB1, and DEFB2, by corticosteroids, hormones that control epididymal function (DUITS *et al*., 2001). Related to this is the fact that two β-defensin-like peptide sequences (HE2ß and HE2γ) are

members of an epididymis-specific family of androgen-regulated proteins (HAMIL *et al*., 2000). In the rat, androgens can regulate the expression of RBD1 in the initial segment of epididymis and caput, but not in corpus or cauda (PALLADINO *et al*., 2003). These authors suggested an antimicrobial role in the testis and epididymis.

The expression of the three new β-defensin genes mapping on chromosome 20 was tested in cell lines from different organs, including those which exhibited expression for other previously described β-defensins. The tested cell lines showed no basal expression (Table 15). These results suggest that their expression is confined to a certain and specific cell type. It may also be due to the fact that, since defensin expression is widely androgen-dependent, the genes might be down-regulated under cell culturing conditions.

4.3 Cellular location of defensins in epididymis

Previous evidence demonstrated that hCAP-18 is located in the epithelium of human epididymis and in seminal fluid. This human cationic peptide was described to be attached to spermatozoa, assuming a role in conception (MALM *et al.*, 2000).

The evaluation of tissue distribution achieved in this work reflects the importance of another group of cationic peptides, the ß-defensins in the male reproductive tract. To localize their specific sites of expression, *in situ* hybridization was performed in caput and caudal regions of the adult human epididymis as well as in lobuli testis.

The present work demonstrates that the mRNA of DEFB29 is restricted to the epididymal duct epithelium of human caput epididymis (Figure 28). As proposed, DEFB1 was located in epithelial cells of human secretory glands (ZHAO *et al*., 1996). A recent publication demonstrated that in mice, the hybridization signal of the mBD-12 antisense probe is also confined to the epithelial cells of the epididymis caput mid/distal segment. No signal for mBD12 was detected in the corpus or caudal region (YAMAGUCHI *et al.,* 2002). *In situ* hybridization showed that HE2 (β-defensin-like peptide) was present exclusively in the epithelial cell layer of the epididymal duct but not within the lumen, nor in peritubular muscle, nor in intertubular tissue (OSTERHOFF *et al*., 1994). Epithelial cells represent more than 80% of the cell population of the epididymal epithelium and play an important role in luminal protein secretion (HERMO *et al.*, 1994). Due to the fact that the novel β-defensins contain secretory signal peptides, it is reasonable to speculate that they are secreted into the epididymal lumen wherein the sperm migrate.

Epididymis is anatomically continuous to the urethra and is always at risk of ascending microbes. Thereby these cells may express and produce the necessary antimicrobial peptides to protect the spermatozoa from invasive infections.

Besides storage, a major function of the epididymis and its corresponding epithelial cells is the disposal of factors to create a microenvironment enabling the spermatozoa to bind and to fertilize eggs, a process which is known as maturation (CORNWALL *et al.*, 1995). Without these specific alterations, spermatozoa lack forward motility and the capability to fertilize (YANAGIMACHI *et al.*, 1994). Thus, it is possible that the epithelial cells express, synthesize and secrete β-defensins to contribute to this process.

In situ hybridization experiments performed for DEFB29 in lobuli testis show a signal with the antisense probe (Figure 30). Nevertheless, these results were not conclusive since a weaker staining was also detected in sections treated with the sense probe (Figure 30). Some genes from the testis as well as other tissues may be transcribed in both directions, creating both sense and antisense RNA (Knee *et al.*, 1994; Ivell *et al.*, 1998). Moreover, this result might be reinforced by the fact that hBD1 was immunolocated in the testicular interstitial compartment (Leydig cells) and in seminiferous tubules (pachytene spermatocytes and spermatogonia) (COM *et al.*, 2003).

4.4 Immunolocation of human β-defensins in the male reproductive tract

Using a monoclonal anti-hBD4 antibody (L1310G6), hBD4 was located in the male genital organ. The peptide was detected by immunohistochemistry in three different locations (Figure 32 and Figure 33):

First, the peptide was located in basal regions of epithelial cells of human epididymis. The endoplasmatic reticulum (synthesis of peptides) and Golgi apparatus (storage of peptides) are in the direct vicinity of the nucleus which is basally located within these cells.

Second, the peptide was detected in cytoplasmic spots of epithelial cells. According to other reports these accumulations might be cytoplasmic vesicles (GLICK and MALHOTRA, 1998).

Third, the peptide was observed surrounding the sperm contained in the epididymal lumen.

These discoveries suggest that the trajectory of the peptide begins in the endoplasmatic reticulum and is subsequently stored or modified in the Golgi apparatus. The vesicles migrate from the perinuclear basal region, through the cytoplasm and after evagination of the cytoplasmic membrane, the peptide are secreted into the lumen wherein the sperm migrate. This theory is in agreement with previous reports (NOVICK *et al.*, 1981).

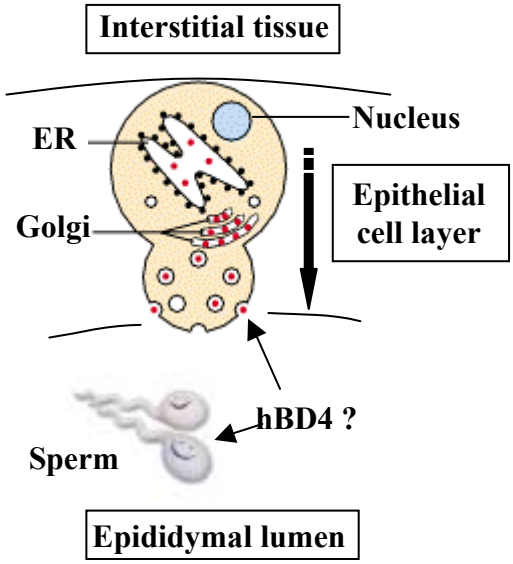


Figure 42. Putative subcellular trafficking of hBD4 in epithelial cells of human caput epididymis.
Proteins destined to be secreted move through the secretory pathway in the following order: rough ER → Golgi cisternae → secretory or transport vesicles → cell surface (exocytosis) →epididymal lumen (wherein the sperm migrate). Discontinuous arrow indicates the putative progression of the peptide.

The first location of the immunostaining was the basal position in epithelial cells of human epididymis. This result is in agreement with other reports that demonstrate the perinuclear location of HE2β1 (β-defensin-like peptide) (HAMIL *et al*., 2000). The authors relate the basal location with the position of the Golgi complex. In contrast, one antibody (P4) specifically directed against HE2β1/HE2E (β-defensin-like peptides) located the peptides in the apical part of the epididymal epithelium (VON HORSTEN *et al.,* 2002).

ESP.13.2, the *Macaca fascicularis* homologue of DEFB26, is stored in the principal cells of the epididymal epithelium. In this case immunofluorescence was present in both apical and basal locations (PERRY *et al.*, 1999).

A second positive staining for hBD4 was observed in apical cytoplasmic spots within the epithelial cells close to the lumen. These cytoplasmic accumulations might be vesicles that transport or secrete peptides (GLICK and MALHOTRA, 1998). The sperm migrate within the lumen, wherein presumably the peptide is supposed to be released by these vesicles. It was observed that early sections of proximal caput epididymis contained less stained vesicles (Figure 32) in comparison to distal caput epididymis (Figure 33). The proximal segment is closer to rete testis, whereas the distal segment of caput epididymis is closer to the corpus epididymis. It has been demonstrated that corpus epididymis presents a much higher expression level of DEFB4 than rete testis (RODRIGUEZ-JIMENEZ *et al*., 2003). These results suggest a correlation between the transcriptional level of DEFB4 along caput epididymis and the biosynthesis of hBD4. To

know if these vesicles are involved in exo- or endocytosis, electron microscopy would be required.

The considered third location for hBD4 is around the sperm, which moves along the epididymal tubular duct. This result is supported by other investigations: HE2ß1 (β-defensin-like peptide) was shown to be expressed in epithelial cells and was observed to be specifically bound to the sperm surface in the postacrosomal and neck regions (HAMIL *et al.*, 2000). The immunostaining of hBD1 was also detected on the head portion of spermatozoa (COM *et al.*, 2003). This is a rather unexpected fact for an antibacterial peptide involved in the innate immunity, unless it is protecting the sperm.

To test the selectivity of the antibodies, a preabsorption control was included in immunohistochemical studies. Nevertheless, this absorption control cannot determine whether the protein bound to the tissue is the same protein that is used for preabsorption. This control consists in a preabsorption of the synthetic peptide used for immunization of the animal with the resultant monoclonal antibody. The expected result would be to detect less signal, since the recognition of the peptide by the antibody should leave less antibody available for staining on the specimen. However, the absorption control can produce staining even though the antibody is specific for the peptide or protein. A complete inhibition of antibody binding to the tissue is difficult. For instance, a small but significant dissociation of the complex antigen-antibody occurs during the incubation.

The interpretation of the results obtained after the preabsorption experiment (Figure 34) is controversial. The preabsorption might create an amplification system. This idea is also supported by other investigators. For instance, it was proposed that albeit unbound primary antibody could have provided the signal, the majority of the labeling occurred because the antibody-protein complex bound to the tissue (Burry, 2000). The author proposed that, after antibody binding, other parts of the protein are available to interact with cytoplasmic proteins in the tissue sections. Then, the protein linked to the antibody binds to the tissue by protein-protein interactions. The strength of this complex could be stronger than a simple protein-antibody binding. When an antibody binds to two separate proteins, the reactions of protein-antibody are thermodynamically slower than those occurring with single proteins. This fact favors protein-protein interactions in the tissue. Thereby, the probability of interacting with endogenous proteins is higher and would explain why the distribution of the endogenous protein is amplified.

The presence of anti-sperm antibodies (ASAs) has been associated with infertility (RUMKE *et al.,* 1959; BRONSON *et al*., 1984). ASAs may impair the function of sperm by recognizing antigens involved in fertility (BRONSON *et al*., 1987). The identification of the antigens to which ASAs are directed is important for understanding their importance in fertilization. One of these antigens located in rat epididymis was recently identified and named E3 (RAO *et al*., 2003). This peptide, with the characteristic six cysteine motif of β-defensins has been considered as a potential element in fertility (RAO *et al*., 2003). The analysis of the secondary structure in mature E3 showed a terminal alpha helical barrel followed by three β-sheets. This structure is also shared by hBD2. These similarities reinforce speculations on the alleged role in fertility of β-defensins.
The fertilization process is the result of a complex sequence of events that collectively result in the fusion of the opposite gametes. The male gamete undergoes continuous morphological and biochemical modifications during sperm development in the testis, maturation in the epididymis, and capacitation in the female reproductive tract. Only capacitated spermatozoa are able to recognize and bind to the ovum's extracellular coat, the zona pellucida (ABOU-HAILA and TULSIANI, 2000). The capacitation and binding processes require the presence of proteins and glycoproteins and their receptors on the surface of the zona pellucida (YANAGIMACHI, 1994). An increase in the fluidity of the plasma membrane of sperm cells could facilitate the installation on the surface of spermatozoa of new proteins and glycoproteins during the epididymal transit (COOPER, 1995). The exocytosis of the acrosomal contents of sperm cells is believed to be a prerequisite that allows the spermatozoa to penetrate the zona pellucida and fertilize the ovum (TULSIANI, 2000). It remains to be shown whether the newly described β-defensins bind to spermatozoa. In this context, it is interesting to note that hBD-3 is capable of inducing reversible ion currents in *Xenopus laevis* oocytes (GARCIA *et al.,* 2001-A). At high concentrations, antimicrobial peptides exhibit more antibiotic activity than cytotoxicity for murine oocytes, and this could be related to the natural, sperm cell-induced perforation of the oocyte membrane (SAWICKY and MYSTKOWSKA, 1999). However, the functional significance of all these findings remains to be determined. To study whether these peptides create a suitable microenvironment to store the sperm under optimal conditions, act on the membrane of germinal cells or have influence on sperm-motility, a combination of β-defensins would be advisable.

4.5 Antimicrobial activity

Defensins have been reported to exhibit powerful antiviral, antifungal, and antibacterial activity against a wide variety of microorganisms (LEHRER *et al.*, 1985; WEINBERG *et al.*, 1998; LEHRER *et al.*, 1989). The innate immune response comprises the first line of host defense against pathogenic microorganisms and it is commonly believed that β-defensins are components of this immunity as a characteristic that identifies them.

It has been demonstrated that human β-defensins e.g. hBD1 (VALORE *et al.,* 1998), hBD2 (TOMITA *et al.,* 2000), hBD3 (GARCIA *et al.*, 2001-A), and hBD4 (GARCIA *et al.*, 2001-B) are antimicrobial agents. The mechanism by which defensins act as antibacterial agents has been analyzed previously (see 1.2) and seems to involve the bacterial cell membrane and intracellular targets (XIONG *et al.*, 1999).

Because the epididymis is anatomically continuous to the urethra, it is always at the risk of ascending microbial invasion. Acute epididymitis is a common sexually transmitted disease, caused by bacterial infection of the epididymis (YAMAGUCHI *et al*., 2002). The mechanisms that protect genitourinary organs from ascending infection by microbes are only partially understood. Since most mucosal surfaces do not normally contain abundant phagocytic cells, one would expect that epithelial cells and their secretions directly interfere with microbial colonization and invasion. The fight against urethral ascending bacterial infections in the male genital organ is very important, because this infection is a frequent cause of infertility after epididymitis. Therefore, host defense against bacterial pathogens would be very important in the epididymis for the protection of spermatozoa. The presence of antimicrobial substances in the epididymis, the major storage site of spermatozoa, might be related to germ cell protection from invading or sexually transmitted microorganisms. Bin1b (the rat homologue of HE2E) shares the β-defensin-specific cysteine pattern and was shown to be expressed in caput epididymis (LI *et al.,* 2001). For this protein, experimental evidence of an antimicrobial effect was provided, and it was assumed that Bin1b prevents the ascent of microorganisms into the adjacent testis.

Other β-defensins show expression in the male reproductive organ. In fact, the expression of hBD1 was determined in seminiferous tubules, to be precise in spermatogonia, pachytene spermatocytes and early spermatids. The peptide was located by immunohistochemistry on the head of the sperm (COM *et al*., 2003). The authors suggested that the spermatozoa contribute to their own defense in the male and female reproductive tract. Moreover, hBD4 is expressed in the male genital organ and exhibits antimicrobial activity (GARCIA *et al*., 2001-B). DEFB23 and

DEFB29 are expressed in epididymal tissue and the synthetic peptides, corresponding to the predicted mature peptides, showed antimicrobial activity. These peptides may be involved in sperm protection from microbial pathogens.

The composition of the amino acid sequences might be crucial for their antibacterial activity and spectra. The enhanced microbicidal activity observed for α-defensins (HNP), with two basic residues at both the N- and C-termini, could be due to optimization of the amphiphilicity of the structure, which could facilitate specific interactions with the microbial membranes (RAJ *et al.*, 2000).

However, these aspects do not explain the differences in the antimicrobial activity of the new human β-defensins. In fact, the hBD23 and hBD29 (synthetic peptide) contain an equivalent number of acid residues at N- and C- termini in comparison to hBD27 (Figure 18). Nevertheless, hBD23 and hBD29 presented antimicrobial activity whereas hBD27 was completely inactive against the tested germs (see 3.11).

On the other hand, it has been suggested that a highly positive net charge in the cysteine core region is a prerequisite for antimicrobial action (HWANG *et al.*, 1998). Synthetic hBD23 contains the most positive charge within the cysteine motif, reflecting more efficient antimicrobial activity. The synthetic hBD29 contains less amino acids with positive charge within this region (Figure 18), presenting a weaker antimicrobial activity. Moreover, hBD27 comprises the least positive charge and is completely inactive against the tested germs. However, with the present results it is not possible to affirm that hBD27 is not an antimicrobial agent. This peptide might show efficiency against other non-tested bacteria, but the results lead to the conclusion that hBD27 is not a broad spectrum antibiotic. Interestingly, following the cysteine core, the additional C-terminal prolongations of hBD27 and hBD29 contain more acidic than basic residues (Figure 18), providing an overall negative charge. From the antimicrobial point of view, this aspect would not explain the presence of such long extensions. The influence of these elongations in the biological effect of the new β-defensins still remains unknown. The results do not permit a unique evaluation of their meaning, since different influences *in vivo* like peptide induction, synergism, and salt dependency might play an important role in the final effect.

The selective toxicity of antibiotic peptides is thought to be due to the composition of the bacterial membrane which contains anionic phospholipids. In contrast, the presence of zwitterionic phospholipids and cholesterol in mammalian cell membranes could prevent interaction with the peptides and avoid host tissue damage (BOMAN *et al.*, 1993; BOMAN *et al.*, 2000, MATZUSAKI *et al.*, 1999). However, it has also been demonstrated that antimicrobial peptides can damage eukaryotic cells (LEHRER *et al.*, 1992; LEHRER *et al.*, 1993; LEHRER *et al.*,

1994). It is commonly believed that the activity of the β-defensin family as antimicrobial agents is based on their interaction with bacterial membranes (LEHRER *et al.*, 1992). However, defensins appear to be electrostatically specific for prokaryotic cells (WEINBERG *et al.*, 1996). Nevertheless, it is of interest to know how the new peptides interact with human cells e.g. erythrocytes. The evaluation of the hemolytic activity shows different results for hBD23 (some hemolytic effect) and hBD27 (no significant hemolytic effect). Since hBD27 does not kill bacteria or destroy erythrocytes, it is possible that hBD27 lacks the ability to disrupt membranes. This peptide seems to lack antimicrobial activity. The ability to act as antimicrobial agents has been considered as the hallmark that characterizes this family of cationic peptides. Moreover, the new members of the β-defensin family are distinctively expressed or located in human epididymis, an organ involved in storage and maturation of sperm. Therefore, the present work introduces new perspectives that can also be of interest for researchers with expertise in fields beyond innate immunity.

5. Bibliography

ABOU-HAILA, A. and TULSIANI, D.R. (2000) Mammalian sperm acrosome: formation, contents, and function. Arch. Biochem. Biophys. **379**: 173-82.

ALTSCHUL, S.F., MADDEN, T.L., SCHAFFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W., LIPMAN, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**: 3389-3402.

ANDREU, D. and RIVAS, L. (1998) Animal antimicrobial peptides: an overview. Biopolymers **47**: 415-33.

ARONSON, S.M. (1994) The miraculous willow tree. R. I. Med. **77**: 159-61.

BALS, R., WANG, X., MEEGALLA, R.L., WATTLER, S., WEINER, D.J., NEHLS, M.C., WILSON, J.M. (1999) Mouse beta-defensin 3 is an inducible antimicrobial peptide expressed in the epithelia of multiple organs. Infect. Immun. **67**: 3542-7.

BALS, R., WANG, X., WU, Z., FREEMAN, T., BAFNA, V., ZASLOFF, M., WILSON, J.M. (1998) Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. J. Clin. Invest. **102**: 874-80.

BECKER, M.N., DIAMOND, G., VERGHESE, M.W., RANDELL, S.H. (2000) CD14-dependent lipopolysaccharide-induced beta-defensin-2 expression in human tracheobronchial epithelium. J. Biol. Chem. **275**: 29731-6.

BEDFORD, J.M. (1994) The status and the state of the human epididymis. Hum. Reprod. **9**: 2187-99.

BENSCH, K.W., RAIDA, M., MAGERT, H.J., SCHULZ-KNAPPE, P., FORSSMANN, W.G. (1995) hBD-1: a novel beta-defensin from human plasma. FEBS Lett. **368**: 331-5.

BEVINS, C.L. and DIAMOND, G. (1996) Molecular biological strategies in the analysis of antibiotic peptide gene families. The use oligonucleotides as hybridization probes. Methods Mol. Biol. **78**: 151-66.

BIRNBOIM, H.C. and DOLY, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**: 1513-22.

BOHEIM, G. (1974) Statistical analysis of alamethicin channels in black lipid membranes. J. Membr. Biol. **19**: 277-303.

BOMAN, H.G. (1993) Antimicrobial peptides. Chairman's opening remarks. Ciba Found Symp. **186**: 1-4.

BOMAN, H.G. (2000) Innate immunity and the normal microflora. Immunol. Rev. **173**: 5-16.

BOS, J.D., PASCH, M.C., ASGHAR, S. (2001) Defensins and complement systems from the perspective of skin immunity and autoimmunity. Clin. Dermatol. **19**: 563-72.

BRONSON, R., COOPER, G., ROSENFELD, D. (1984) Sperm antibodies their role in infertility. Fertl. Steril. **42**: 171-83.

BRONSON, R. (1987) Immunity in sperm and in vitro fertilization. J. In Vitro Fert. Embryo Transf. **4**: 195-7.

BURRY, W.R. (2000) Specificity controls for immunocytochemical methods. J. Histochem. Cytochem. **48**: 163-5.

CHERTOV, O., MICHIEL, D.F., XU, L., WANG, J.M., TANI, K., MURPHY, W.J., LONGO, D.L., TAUB, D.D., OPPENHEIM, J.J. (1996) Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. J. Biol. Chem. **271**: 2935-40.

CHILDS, G.V. and UNABIA, G. (1982) Application of a rapid avidin--biotin--peroxidase complex (ABC) technique to the localization of pituitary hormones at the electron microscopic level. J. Histochem. Cytochem. **30**: 1320-4.

CHRONNELL, C.M., GHALI, L.R., ALI, R.S., QUINN, A.G., HOLLAND, D.B., BULL, J.J., CUNLIFFE, WJ., MCKAY, I.A., PHILPOTT, M.P., MULLER-ROVER, S. (2001) Human beta defensin-1 and -2 expression in human pilosebaceous units: upregulation in acne vulgaris lesions. J. Invest. Dermatol. **117**: 1120-5.

COLE, A.M. and GANZ, T. (2000) Human antimicrobial peptides: analysis and application. Biotechniques **29**: 822-30.

COM, E., BOURGEON, F., EVRARD, B., GANZ, T., COLLEU, D., JEGOU, B., PINEAU, C. (2003) Expression of antimicrobial defensins in the male reproductive tract of rats, mice, and humans. Biol. Reprod. **68**: 95-104.

COOPER, T.G., (1995) Role of the epididymis in mediating changes in the male gamete during maturation. Adv. Exp. Med. Biol. **377**: 87-101.

COOPER, T.G., WAITES, G.M., NIESCHLAG, E. (1986) The epididymis and male fertility. A symposium report. Int. J. Androl. **9**: 81-90.

CORNWALL, G.A. and HANN, S.R. (1995) Specialized gene expression in the epididymis. J. Androl. **16**: 379-83.

CORNWALL, G.A. and HANN, S.R. (1995) Transient appearance of CRES protein during spermatogenesis and caput epididymal sperm maturation. Mol. Reprod. Dev. **41**: 37-46.

COUTO, M.A., HARWIG, S.S., CULLOR, J.S., HUGHES, J.P., LEHRER, R.I. (1992) Identification of eNAP-1, an antimicrobial peptide from equine neutrophils. Infect. Immun. **60**: 3065-71.

DE KRETSER, D.M., LOVELAND, K.L., MEINHARDT, A., SIMORANGKIR, D., WREFORD, N. (1998) Spermatogenesis. Hum. Reprod. **13**: 1-8.

DIAMOND, G., KAISER, V., RHODES, J., RUSSELL, J.P., BEVINS, C.L. (2000) Transcriptional regulation of beta-defensin gene expression in tracheal epithelial cells. Infect. Immun. **68**: 113-9.

DIAMOND, G., RUSSELL, J.P., BEVINS, C.L. (1996) Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. Proc. Natl. Acad. Sci. USA **93**: 5156-60.

DIAMOND, G., ZASLOFF, M., ECK, H., BRASSEUR, M., MALOY, W.L., BEVINS, C.L. (1991) Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. Proc. Natl. Acad. Sci. USA **88**: 3952-6.

DIMARCQ, J.L., HOFFMANN, D., MEISTER, M., BULET, P., LANOT, R., REICHHART, J.M., HOFFMANN, J.A. (1994) Characterization and transcriptional profiles of a Drosophila

gene encoding an insect defensin. A study in insect immunity. Eur. J. Biochem. **221**: 201-9.

DUITS, L.A., RADEMAKER, M., RAVENSBERGEN, B., VAN STERKENBURG, M.A., VAN STRIJEN, E., HIEMSTRA, P.S., NIBBERING, P.H. (2001) Inhibition of hBD-3, but not hBD-1 and hBD-2, mRNA expression by corticosteroids. Biochem. Biophys. Res. Commun. **280**: 522-5.

EISENHAUER, P.B., HARWIG, S.S., LEHRER, R.I. (1992) Cryptdins: antimicrobial defensins of the murine small intestine. Infect. Immun. **60**: 3556-65.

EISENHAUER, P.B., HARWIG, S.S., SZKLAREK, D., GANZ, T., SELSTED, M.E., LEHRER, R.I. (1989) Purification and antimicrobial properties of three defensins from rat neutrophils. Infect. Immun. **57**: 2021-7.

EPPLE, P., APEL, K., BOHLMANN, H. (1997) ESTs reveal a multigene family for plant defensins in Arabidopsis thaliana. FEBS Lett. **400**: 168-72.

FANG, X.M., SHU, Q., CHEN, Q.X., BOOK, M., SAHL, H.G., HOEFT, A., STUBER, F. (2003) Differential expression of alpha- and beta-defensins in human peripheral blood. Eur. J. Clin. Invest. **33**: 82-7.

FROHLICH, O., PO, C., MURPHY, T., YOUNG, L.G. (2000) Multiple promoter and splicing mRNA variants of the epididymis-specific gene EP2. J. Androl. **21**: 421-30.

FROHLICH, O., PO, C., YOUNG, L.G. (2001) Organization of the human gene encoding the epididymis-specific EP2 protein variants and its relationship to defensin genes. Biol. Reprod. **64**: 1072-9.

FROHMAN, M.A., DUSH, M.K., MARTIN, G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85**: 8998-9002.

GANZ, T., SELSTED, M.E., LEHRER, R.I. (1990) Defensins. Eur. J. Haematol. **44**: 1-8.

GANZ, T., SELSTED, M.E., SZKLAREK, D., HARWIG, S.S., DAHER, K., BAINTON, D.F., LEHRER, R.I. (1985) Defensins. Natural peptide antibiotics of human neutrophils. J. Clin. Invest. **76**: 1427-35.

GARCIA, J.R., JAUMANN, F., SCHULZ, S., KRAUSE, A., RODRIGUEZ-JIMENEZ, J., FORSSMANN, U., ADERMANN, K., KLUVER, E., VOGELMEIER, C., BECKER, D., HEDRICH, R., FORSSMANN, W.G., BALS, R. (2001) Identification of a novel, multifunctional beta-defensin (human beta-defensin 3) with specific antimicrobial activity. Its interaction with plasma membranes of Xenopus oocytes and the induction of macrophage chemoattraction. Cell Tissue Res. **306**: 257-64. This paper is cited: (GARCIA *et al*., 2001-A).

GARCIA, JR., KRAUSE, A., SCHULZ, S., RODRIGUEZ-JIMENEZ, F.J., KLUVER, E., ADERMANN, K., FORSSMANN, U., FRIMPONG-BOATENG, A., BALS, R., FORSSMANN, W.G. (2001) Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. FASEB J. **15**: 1819-21. This paper is cited: (GARCIA *et al*., 2001-B).

GLICK, B.S. and MALHOTRA, V. (1998) The curious status of the Golgi apparatus. Cell. **95**: 883-9.

GOUGH, M., HANCOCK, R.E., KELLY, N.M. (1996) Antiendotoxin activity of cationic peptide antimicrobial agents. Infect. Immun. **64**: 4922-7.

GOULD, K.G. and YOUNG, L.G. (1990) Acquisition of fertilizing capacity by chimpanzee sperm. Folia Primatol (Basel) **54**: 105-8.

GRAY, W.R. (1993) Echistatin disulfide bridges: selective reduction and linkage assignment. Protein Sci. **2**: 1749-55.

GUDMUNDSSON, G.H., AGERBERTH, B., ODEBERG, J., BERGMAN, T., OLSSON, B., SALCEDO, R. (1996) The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. Eur. J. Biochem. **238**: 325-32.

HAMIL, K.G., SIVASHANMUGAM, P., RICHARDSON, R.T., GROSSMAN, G., RUBEN, S.M., MOHLER, J.L., PETRUSZ, P., O'RAND, M.G., FRENCH, F.S., HALL, S.H. (2000) HE2beta and HE2gamma, new members of an epididymis-specific family of androgen-regulated proteins in the human. Endocrinology **141**: 1245-53.

HAMILTON, P. (1990) Sperm banking before chemotherapy or radiotherapy for cancer. N Engl. J. Med. **322**: 1822.

HANCOCK, R.E. and LEHRER, R. (1998) Cationic peptides: a new source of antibiotics. Trends. Biotechnol. **16,** (2):82-8.

HANCOCK, R.E. and SCOTT, M.G. (2000) The role of antimicrobial peptides in animal defenses. Proc. Natl. Acad. Sci. USA **97**: 8856-61.

HARDER, J., BARTELS, J., CHRISTOPHERS, E., SCHRODER, J.M. (2001) Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. J. Biol. Chem. **276**: 5707-13.

HARDER, J., BARTELS, J., CHRISTOPHERS, E., SCHRODER, J.M. (1997) A peptide antibiotic from human skin. Nature **387**: 861.

HARDER, J., SIEBERT, R., ZHANG, Y., MATTHIESEN, P., CHRISTOPHERS, E., SCHLEGELBERGER, B., SCHRODER, J.M. (1997) Mapping of the gene encoding human beta-defensin-2 (DEFB2) to chromosome region 8p22-p23.1. Genomics. **46**: 472-5.

HARWIG, S.S., SWIDEREK, K.M., KOKRYAKOV, V.N., TAN, L., LEE, T.D., PANYUTICH, E.A., ALESHINA, G.M., SHAMOVA, O.V., LEHRER, R.I. (1994) Gallinacins: cysteine-rich antimicrobial peptides of chicken leukocytes. FEBS Lett. **342**: 281-5.

HE, K., LUDTKE, S.J., HELLER, W.T., HUANG, H.W. (1996) Mechanism of alamethicin insertion into lipid bilayers. Biophys. J. **71**: 2669-79.

HELMERHORST, E.J., REIJNDERS, I.M., VAN 'T HOF, W., VEERMAN, E.C., NIEUW AMERONGEN, A.V. (1999) A critical comparison of the hemolytic and fungicidal activities of cationic antimicrobial peptides. FEBS Lett. **449**: 105-10.

HERMO, L., OKO, R., MORALES, C.R. (1994) Secretion and endocytosis in the male reproductive tract: a role in sperm maturation. Int. Rev. Cytol. **154**: 106-89.

HIGAZI, A.A., GANZ, T., KARIKO, K., CINES, D.B. (1996) Defensin modulates tissue-type plasminogen activator and plasminogen binding to fibrin and endothelial cells. J. Biol. Chem. **271**: 17650-5.

HINTON, B.T., PALLADINO, M.A., RUDOLPH, D., LAN, Z.J., LABUS, J.C. (1996) The role of the epididymis in the protection of spermatozoa. Curr. Top. Dev. Biol. **33**: 61-102.

HOLLAND, P.M., ABRAMSON, R.D., WATSON, R., GELFAND, D.H. (1991) Detection of specific polymerase chain reaction product by utilizing the 5′ to 3′ exonuclease activity of Thermus aquaticus DNA polymerase. Proc. Natl. Acad. Sci. USA **88**: 7276-80.

HOLSTEIN, A.F. (1969) On the problem of the local control of the spermatogenesis of the spiny dogfish (Squalus acanthias L. Z Zellforsch Mikrosk. Anat. **93**: 265-81.

HUH, W.K., OONO, T., SHIRAFUJI, Y., AKIYAMA, H., ARATA, J., SAKAGUCHI, M., HUH, N.H., IWATSUKI, K. (2002) Dynamic alteration of human beta-defensin 2 localization from cytoplasm to intercellular space in psoriatic skin. J. Mol. Med. **80**: 678-84.

HUTTNER, K.M., KOZAK, C.A., BEVINS, C.L. (1997) The mouse genome encodes a single homolog of the antimicrobial peptide human beta-defensin 1. FEBS Lett; **413**: 45-9.

HUTTNER, K.M., LAMBETH, M.R., BURKIN, H.R., BURKIN, D.J., BROAD, T.E. (1998) Localization and genomic organization of sheep antimicrobial peptide genes. Gene. **206**: 85-91.

HWANG, P.M. and VOGEL, H.J. Structure-function relationships of antimicrobial peptides. Biochem. Cell. Biol. **76**: 235-46.

ISENBERG, H.D. (1993) Clinical microbiology procedures handbook, Supplement 1. American Society for Microbiology, New York.

IVELL, R. (1998) A question of faith-or the philosophy of RNA controls. J. Endocrinol. **159**: 197-200.

JIA, H.P., SCHUTTE, B.C., SCHUDY, A., LINZMEIER, R., GUTHMILLER, J.M., JOHNSON, G.K., TACK, B.F., MITROS, J.P., ROSENTHAL, A., GANZ, T., McCRAY, P.B. (2001) Discovery of new human beta-defensins using a genomics-based approach. Gene **263**: 211-8.

JIA, H.P., WOWK, S.A., SCHUTTE, B.C., LEE, S.K., VIVADO, A., TACK, B.F., BEVINS, C.L., MCCRAY, P.B. (2000) A novel murine beta -defensin expressed in tongue, esophagus, and trachea. J. Biol. Chem. **275**: 33314-20.

JONES, R. (1998) Plasma membrane structure and remodelling during sperm maturation in the epididymis. J. Reprod. Fertil. Suppl. **53**: 73-84.

JONES, R.C. (1998) Evolution of the vertebrate epididymis. J. Reprod. Fertil. Suppl. **53**: 163-81.

KAISER, V. and DIAMOND, G. (2000) Expression of mammalian defensin genes. J. Leukoc. Biol. **68**: 779-84.

KING, D. (2002) http://www.siumed.edu/~dking2/erg/epidid.htm.

KING, A.E., FLEMING, D.C., CRITCHLEY, H.O., KELLY, R.W. (2002) Regulation of natural antibiotic expression by inflammatory mediators and mimics of infection in human endometrial epithelial cells. Mol. Hum. Reprod. **8**: 341-9.

KIRCHHOFF, C. (1999) Gene expression in the epididymis. Int. Rev. Cytol. **188**: 133-202.

KNEE, R.S., PITCHER S.E., MURPHY, P.R. (1994) Basic fibroblast growth factor sense (FGF) and antisense (GFG) RNA transcripts are expressed in unfertilized human oocytes and in differentiated adult tissues. Biochem. Biophys. Res. Communic. **205**: 577-83.

KRULL, N., IVELL, R., OSTERHOFF, C., KIRCHHOFF, C. (1995) Region-specific variation of gene expression in the human epididymis as revealed by in situ hybridization with tissue-specific cDNAs. Mol. Reprod. Dev. **34,** (1):16-24.

LARRICK, J.W., HIRATA, M., BALINT, R.F., LEE, J., ZHONG, J., WRIGHT, S.C. (1995) Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. Infect. Immun.**63**: 1291-7.

LEE, L.G., CONNELL, C.R., BLOCH, W. (1993) Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. **21**: 3761-6.

LEHRER, R.I., BARTON, A., DAHER, K.A., HARWIG, S.S., GANZ, T., SELSTED, M.E. (1989) Interaction of human defensins with Escherichia coli. Mechanism of bactericidal activity. J. Clin. Invest. **84**: 553-61.

LEHRER, R.I., DAHER, K., GANZ, T., SELSTED, M.E. (1985) Direct inactivation of viruses by MCP-1 and MCP-2, natural peptide antibiotics from rabbit leukocytes. J. Virol. **54**: 467-72.

LEHRER, R.I. and GANZ, T. (1999) Antimicrobial peptides in mammalian and insect host defense. Curr. Opin. Immunol. **11**: 23-7.

LEHRER, R.I., LICHTENSTEIN, A.K., GANZ, T. (1993) Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu. Rev. Immunol. **11**: 105-28.

LEHRER, R.I., ROSENMAN, M., HARWIG, S.S.L., JACKSON, R. EISENHAUER, P. (1991) Ultrasensitive assays for endogenous antimicrobial polypeptides. J. Immun. Methods **137**: 167-73.

LEMAITRE, B., REICHHART, J.M., HOFFMANN, J.A. (1997) Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc. Natl. Acad. Sci. USA **94**: 14614-9.

LI, P., CHAN, H.C., HE, B., SO, S.C., CHUNG, Y.W., SHANG, Q., ZHANG, Y.D., ZHANG, Y.L. (2001) An antimicrobial peptide gene found in the male reproductive system of rats. Science **291**: 1783-5.

LILLARD, J.W., BOYAKA, P.N., CHERTOV, O., OPPENHEIM, J.J., MCGHEE, J.R. (1999) Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. Proc. Natl. Acad. Sci. USA **96**: 651-6.

LINDEMANN, R.A., LALA, A., MIYASAKI, K.T. (1994) The in vitro effect of human polymorphonuclear leukocyte azurophil granule components on natural killer cell cytotoxicity. Oral Microbiol. Immunol. **9**: 186-92.

LIU, L., ZHAO, C., HENG, H.H., GANZ, T. (1997) The human beta-defensin-1 and alpha-defensins are encoded by adjacent genes: two peptide families with differing disulfide topology share a common ancestry. Genomics **43**: 316-20.

MALM, J., SORENSEN, O., PERSSON, T., FROHM-NILSSON, M., JOHANSSON, B., BJARTELL, A., LILJA, H., STAHLE-BACKDAHL, M., BORREGAARD, N., EGESTEN, A. (2000) The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa. Infect. Immun. **68**: 4297-302.

MARCHINI, G., LINDOW, S., BRISMAR, H., STABI, B., BERGGREN, V., ULFGREN, AK., LONNE-RAHM, S., AGERBERTH, B., GUDMUNDSSON, G.H. (2002) The newborn infant is protected by an innate antimicrobial barrier: peptide antibiotics are present in the skin and vernix caseosa. Br. J. Dermatol. **147**: 1127-34.

MATSUZAKI, K. and HORIKIRI, C. (1999) Interactions of amyloid beta-peptide (1-40) with ganglioside-containing membranes. Biochemistry **38**: 4137-42.

MCCRAY, P.B. and BENTLEY, L.(1997) Human airway epithelia express a beta-defensin. Am. J. Respir. Cell. Mol. Biol. **16**: 343-9.

MORRISON, G., KILANOWSKI, F., DAVIDSON, D., DORIN, J. (2002) Characterization of the mouse beta defensin 1, Defb1, mutant mouse model. Infect. Immun. **70**: 3053-60.

MORRISON, G.M., DAVIDSON, D.J., DORIN, J.R. (1999) A novel mouse beta defensin, Defb2, which is upregulated in the airways by lipopolysaccharide. FEBS Lett. **442**: 112-6.

MURPHY, C.J., FOSTER, B.A., MANNIS, M.J., SELSTED, M.E., REID, T.W. (1993) Defensins are mitogenic for epithelial cells and fibroblasts. J. Cell. Physiol. **155**: 408-13.

NIEBUHR, K., LINGNAU, A., FRANK, R., WEHLAND, J. (1998) Cell Biology: A Laboratory Handbook. Academic Press, second edition, Vol. **2**: 398-403.

NIELSEN, H., ENGELBRECHT, J., BRUNAK, S., VON HEIJNE, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10**: 1-6.

NISHIMURA, A., MORITA, M., NISHIMURA, Y., SUGINO, Y. (1990) A rapid and highly efficient method for preparation of competent Escherichia coli cells. Nucleic Acids Res. **18**: 6169.

NOLAN, J.P. and HAMMERSTEDT, R.H. (1997) Regulation of membrane stability and the acrosome reaction in mammalian sperm. FASEB J. **11**: 670-82.

NOVICK, P., FERRO, S., SCHEKMAN, R. (1981) Order of events in the yeast secretory pathway. Cell **25**: 461-9.

ONG, P.Y., OHTAKE, T., BRANDT, C., STRICKLAND, I., BOGUNIEWICZ, M., GANZ, T., GALLO, R.L., LEUNG, D.Y. (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N. Engl. J. Med. **347**: 1151-60.

ORGEBIN-CRIST, M.C. (1995) The epididymis across 24 centuries. J. Reprod. Fertil. Suppl. **53**: 285-92.

OSTERHOFF, C., KIRCHHOFF, C., KRULL, N., IVELL, R. (1994) Molecular cloning and characterization of a novel human sperm antigen (HE2) specifically expressed in the proximal epididymis. Biol. Reprod. **50**: 516-25.

OUELLETTE, A.J., MILLER, S.I., HENSCHEN, A.H., SELSTED, M.E. (1992) Purification and primary structure of murine cryptdin-1, a Paneth cell defensin. FEBS. Lett. **304**: 146-8.

OUELLETTE, A.J. (1997) Paneth cells and innate immunity in the crypt microenvironment. Gastroenterology **113**: 1779-84.

PALLADINO, M.A., MALLONGA, T.A., MISHRA, M.S. (2003) Messenger RNA (mRNA) Expression for the Antimicrobial Peptides beta-Defensin-1 and beta-Defensin-2 in the Male Rat Reproductive Tract: beta-Defensin-1 mRNA in Initial Segment and Caput

Epididymidis Is Regulated by Androgens and Not Bacterial Lipopolysaccharides. Biol. Reprod. **68**: 509-15.

PANYUTICH, A.V., SZOLD, O., POON, P.H., TSENG, Y., GANZ, T. (1994) Identification of defensin binding to C1 complement. FEBS Lett. **356**: 169-73.

PERA, I., IVELL, R., KIRCHHOFF, C. (1994) Regional variation of specific gene expression in the dog epididymis as revealed by in-situ transcript hybridization. Int. J. Androl. **17**: 324-30.

PERI, A., FANTONI, G., GRANCHI, S., VANNELLI, G.B., BARNI, T., AMERINI, S., PUPILLI, C., BARBAGLI, G., FORTI, G., SERIO, M., MAGGI, M. (1997) Gene expression of endothelin-1, endothelin-converting enzyme-1, and endothelin receptors in human epididymis. J. Clin. Endocrinol. Metab. **82**: 3797-806.

PERRY, A.C., JONES, R., MOISYADI, S., COADWELL, J., HALL, L. (1999) The novel epididymal secretory protein ESP13.2 in Macaca fascicularis. Biol. Reprod. **61**: 965-72.

QUAYLE, A.J., PORTER, E.M., NUSSBAUM, A.A., WANG, Y.M., BRABEC, C., YIP, K.P., MOK, S.C. (1998) Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. Am. J. Pathol. **152**: 1247-58.

RAJ, P.A., ANTONYRAJ, K.J., KARUNAKARAN, T. (2000) Large-scale synthesis and functional elements for the antimicrobial activity of defensins. Biochem. J. **347**: 633-41.

RAO, A.G., ROOD, T., MADDOX, J., DUVICK, J. (1992) Synthesis and characterization of defensin NP-1. Int. J. Pept. Protein. Res. **40**: 507-14.

RAO, J., HERR, J.C., REDDI, P.P., WOLKOWICZ, M.J., BUSH, L.A., SHERMAN, N.E., BLACK, M., FLICKINGER, C.J. (2003) Cloning and characterization of a novel sperm-associated isoantigen (e-3) with defensin- and lectin-like motifs expressed in rat epididymis. Biol. Reprod. **68**: 290-301.

RODRIGUEZ, C.M., KIRBY, J.L., HINTON, B.T. (2001) Regulation of gene transcription in the epididymis. Reproduction **122**: 41-8.

RODRIGUEZ-JIMENEZ, F.J., KRAUSE, A., SCHULZ, S., FORSSMANN, W.G., CONEJO-GARCIA, J.R., SCHREEB, R., MOTZKUS, D. (2003) Distribution of new human beta-defensin genes clustered on chromosome 20 in functionally different segments of epididymis. Genomics **81**: 175-83.

RUMKE, P. and HELLINGA, G. (1959) Autoantibodies against spermatozoa in sterile men. Am. J. Clin. Pathol. **32**: 357-63.

RUSSELL, J.P., DIAMOND, G., TARVER, A.P., SCANLIN, T.F., BEVINS, C.L. (1996) Coordinate induction of two antibiotic genes in tracheal epithelial cells exposed to the inflammatory mediators lipopolysaccharide and tumor necrosis factor alpha. Infect. Immun. **64**: 1565-8.

SAIKI, R.K., SCHARF, S., FALOONA, F., MULLIS, K.B., HORN, G.T., ERLICH, H.A., ARNHEIM, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**: 1350-4.

SANGER, F., NICKLEN, S. COULSON, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**: 5463-7.

SAWICKI, W. and MYSTKOWSKA, E.T. (1999) Contraceptive potential of peptide antibiotics. Lancet. **353**: 464-5.

SCHRODER, J.M. and HARDER, J. (1999) Human beta-defensin-2. Int. J. Biochem. Cell. Biol. **31**: 645-51.

SELSTED, M.E., SZKLAREK, D., LEHRER, R.I. (1984) Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes. Infect. Immun. **45**: 150-4.

SELSTED, M.E., TANG, Y.Q., MORRIS, W.L., MCGUIRE, P.A., NOVOTNY, M.J., SMITH, W., HENSCHEN, A.H., CULLOR, J.S. (1993) Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils. J Biol. Chem. **268**: 6641-8.

SEO, S.J., AHN, S.W., HONG, C.K., RO, B.I. (2001) Expressions of beta-defensins in human keratinocyte cell lines. J. Dermatol. Sci. **27**: 183-91.

SHI, J., ZHANG, G., WU, H., ROSS, C., BLECHA, F., GANZ, T. (1999) Porcine epithelial beta-defensin 1 is expressed in the dorsal tongue at antimicrobial concentrations. Infect. Immun. **67**: 3121-7.

STOLZENBERG, E.D., ANDERSON, G.M., ACKERMANN, M.R., WHITLOCK, R.H., ZASLOFF, M. (1997) Epithelial antibiotic induced in states of disease. Proc. Natl. Acad. Sci. USA **94**: 8686-90.

SUAREZ, S.S. (1987) Sperm transport and motility in the mouse oviduct: observations in situ. Biol. Reprod. **36**: 203-10.

SÜSSMUTH, R., EBERSPÄCHER, J., HAAG, R., SPRINGER, W. (1999) Mikrobiologisch-biochemisches Praktikum. 2. Auflage, Georg Thieme Verlag Stuttgart.

TANG, YQ., YUAN, J., OSAPAY, G., OSAPAY, K., TRAN, D., MILLER, C.J., OUELLETTE, A.J., SELSTED, M.E. (1999) A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. Science. **286**: 498-502.

TANI, K., MURPHY, W.J., CHERTOV, O., SALCEDO, R., KOH, C.Y., UTSUNOMIYA, I., FUNAKOSHI, S., ASAI, O., HERRMANN, S.H., WANG, J.M., KWAK, L.W., OPPENHEIM, J.J. (2000) Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. Int. Immunol. **12**: 691-700.

TERRITO, M.C., GANZ, T., SELSTED, M.E., LEHRER, R. (1989) Monocyte-chemotactic activity of defensins from human neutrophils. J. Clin. Invest. **84**: 2017-20.

TOMITA, T., HITOMI, S., NAGASE, T., MATSUI, H., MATSUSE, T., KIMURA, S., OUCHI, Y. (2000) Effect of ions on antibacterial activity of human beta defensin 2. Microbiol. Immunol. **44,** (9):749-54.

TRAN, D., TRAN, P.A., TANG, Y.Q., YUAN, J., COLE, T., SELSTED, M.E. (2002) Homodimeric theta-defensins from rhesus macaque leukocytes: isolation, synthesis, antimicrobial activities, and bacterial binding properties of the cyclic peptides. J. Biol. Chem. **277**: 3079-84.

TSUKAMOTO, T., KUSAKABE, M., SAGA, Y. (1991) In situ hybridization with non-radioactive digoxigenin-11-UTP-labeled cRNA probes: localization of developmentally regulated mouse tenascin mRNAs. Int. J. Dev. Biol. **35**: 25-32.

TULSIANI, D.R. (2000) Carbohydrates mediate sperm-ovum adhesion and triggering of the acrosome reaction. Asian J. Androl. **2**: 87-97.

TURNER, T.T. (1995) On the epididymis and its role in the development of the fertile ejaculate. J. Androl. **16**: 292-8.

VALORE, E.V., PARK, C.H., QUAYLE, A.J., WILES, K.R., MCCRAY, P.B., GANZ, T. (1998) Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. J. Clin. Invest. **101**: 1633-42.

VAN WETERING, S., MANNESSE-LAZEROMS, S.P., DIJKMAN, J.H., HIEMSTRA, P.S. (1997) Effect of neutrophil serine proteinases and defensins on lung epithelial cells: modulation of cytotoxicity and IL-8 production. J. Leukoc. Biol. **62**: 217-26.

VAN WETERING, S., MANNESSE-LAZEROMS, S.P., VAN STERKENBURG, M.A., DAHA, M.R., DIJKMAN, J.H., HIEMSTRA, P.S. (1997) Effect of defensins on interleukin-8 synthesis in airway epithelial cells. Am. J. Physiol. **272**: L888-96.

VAN WETERING, S., STERK, P.J., RABE, K.F., HIEMSTRA, P.S. (1999) Defensins: key players or bystanders in infection, injury, and repair in the lung?. J. Allergy. Clin. Immunol. **104**: 1131-8.

VOGELSTEIN, B. and GILLESPIE, D. (1979) Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA **76**: 615-619.

VON HORSTEN, H.H., DERR, P., KIRCHHOFF, C. (2002) Novel antimicrobial peptide of human epididymal duct origin. Biol. Reprod. **67**: 804-13.

WEINBERG, A., KRISANAPRAKORNKIT, S., DALE, B.A. (1998) Epithelial antimicrobial peptides: review and significance for oral applications. Crit. Rev. Oral. Biol. Med. **9**: 399-414.

WILSON, C.L., OUELLETTE, A.J., SATCHELL, D.P., AYABE, T., LOPEZ-BOADO, Y.S., STRATMAN, J.L., HULTGREN, S.J., MATRISIAN, L.M., PARKS, W.C. (1999) Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science **286**: 113-7.

XIONG, Y.Q., YEAMAN, M.R., BAYER, A.S. (1999) In vitro antibacterial activities of platelet microbicidal protein and neutrophil defensin against Staphylococcus aureus are influenced by antibiotics differing in mechanism of action. Antimicrob. Agents Chemother. **43**: 1111-7.

YAGI, A. and PARANKO, J. (1992) Localization of actin, alpha-actinin, and tropomyosin in bovine spermatozoa and epididymal epithelium. Anat. Rec. **233**: 61-74.

YAMAGUCHI, Y., FUKUHARA, S., NAGASE, T., TOMITA, T., HITOMI, S., KIMURA, S., KURIHARA, H., OUCHI, Y. (2001) A novel mouse beta-defensin, mBD-6, predominantly expressed in skeletal muscle. J. Biol. Chem. **276**: 31510-4.

YAMAGUCHI, Y., NAGASE, T., MAKITA, R., FUKUHARA, S., TOMITA, T., TOMINAGA, T., KURIHARA, H., OUCHI, Y. (2002) Identification of multiple novel epididymis-specific beta-defensin isoforms in humans and mice. J. Immunol. **169**: 2516-23.

YANAGIMACHI, R. (1994) Fertility of mammalian spermatozoa: its development and relativity. Zygote. **2**: 371-2.

YANG, D., CHERTOV, O., BYKOVSKAIA, S.N., CHEN, Q., BUFFO, M.J., SHOGAN, J., ANDERSON, M., SCHRODER, J.M., WANG, J.M., HOWARD, O.M., OPPENHEIM, J.J. (1999) Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science. **286**: 525-8.

YELTON, D.E. and SCHARFF, M.D. (1980) Monoclonal antibodies. Am. Sci. **68**: 510-6.

YEUNG, C.H., COOPER, T.G., OBERPENNING, F., SCHULZE, H., NIESCHLAG, E. (1993) Changes in movement characteristics of human spermatozoa along the length of the epididymis. Biol. Reprod. **49**: 274-80.

YOUNT, N.Y., YUAN, J., TARVER, A., CASTRO, T., DIAMOND, G., TRAN, P.A., LEVY, J.N., MCCULLOUGH, C., CULLOR, J.S., BEVINS, C.L., SELSTED, M.E. (1999) Cloning and expression of bovine neutrophil beta-defensins. Biosynthetic profile during neutrophilic maturation and localization of mature peptide to novel cytoplasmic dense granules. J. Biol. Chem. **274**: 26249-58.

ZANETTI, M., GENNARO, R., ROMEO, D. (1995) Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett. **374**: 1-5.

ZHANG, G., WU, H., SHI, J., GANZ, T., ROSS, C.R., BLECHA, F. (1998) Molecular cloning and tissue expression of porcine beta-defensin-1. FEBS Lett. **424**: 37-40.

ZHANG, L., YU, W., HE, T., YU, J., CAFFREY, R.E., DALMASSO, E.A., FU, S., PHAM, T., MEI, J., HO, J.J., ZHANG, W., LOPEZ, P., HO, D.D. (2002) Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. Science **298**: 995-1000.

ZHAO, C., WANG, I., LEHRER, R.I. (1996) Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. FEBS Lett. **396**: 319-22.

ZHU, Q.Z., SINGH, A.V., BATEMAN, A., ESCH, F., SOLOMON, S. (1987) The corticostatic (anti-ACTH) and cytotoxic activity of peptides isolated from fetal, adult and tumor-bearing lung. J. Steroid. Biochem. **27**: 1017-22.

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Publications

Original work:

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GARCIA, J.R., JAUMANN, F., SCHULZ, S., KRAUSE, A., **RODRIGUEZ-JIMENEZ, J**., FORSSMANN, U., ADERMANN, K., KLUVER, E., VOGELMEIER, C., BECKER, D., HEDRICH, R., FORSSMANN, W.G., BALS, R. (2001) Identification of a novel, multifunctional beta-defensin (human beta-defensin 3) with specific antimicrobial activity. Its interaction with plasma membranes of Xenopus oocytes and the induction of macrophage chemoattraction. Cell Tissue Res. **306**: 257-64. This paper is cited: (GARCIA *et al*., 2001-A).

GARCIA, JR., KRAUSE, A., SCHULZ, S., **RODRIGUEZ-JIMENEZ, F.J**., KLUVER, E., ADERMANN, K., FORSSMANN, U., FRIMPONG-BOATENG, A., BALS, R., FORSSMANN, W.G. (2001) Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. FASEB J. **15**: 1819-21. This paper is cited: (GARCIA *et al*., 2001-B).

MORENO-MANZANO, V., SEPÚLVEDA-MUNOZ, J.C., **RODRIGUEZ-JIMENEZ, J**., RODRIGUEZ-PUYOL, M., RODRIGUEZ-PUYOL, D., KITAMURA, M., LUCIO CAZAÑA, F.J. (2000) Human renal mesangial cells are a target for the anti-inflammatory action of 9-cis retinoic acid. Brit. J. Pharmacol **131:** 1673-83.

MORENO-MANZANO, V., MAMPASO, F., SEPULVEDA-MUNOZ, J.C., **RODRIGUEZ, J**., NAVARRO, E., KITAMURA, M., CHEN, S., ZIYADEH, F.N., ORELLANA, J.M., IGLESIAS-DE LA CRUZ, M.C., LUCIO, F.J. Retinoids as a potential treatment for experimental puromycin-induced nephrosis. Brit. J. Pharmacol. (in press).

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Prizes

2000 Real academia de Farmacia. CEPA prize. MORENO MANZANO, M.V., **RODRÍGUEZ JIMÉNEZ, F.J**., RODRÍGUEZ PUYOL, M., MAMPASO MARTÍN-BUITRAGO, F., SEPÚLVEDA MUÑOZ, J.C., LUCIO CAZAÑA, F.J. Acción Antiinflamatoria de los retinoides en la glomeruloesclerosis.

2000 IPF Pharmaceuticals. GARCIA, J.R., KRAUSE, A., SCHULZ, S., **RODRIGUEZ-JIMENEZ, F.J**., KLUVER, E., SCHREEB, R.

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Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass die vorliegende Dissertation von mir selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt wurde. Zu Hilfsleistungen herangezogene Institutionen und Personen habe ich vollständig angegeben. Ich versichere ferner, dass die Dissertation nicht schon als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet worden ist.

Hannover, in Francisco Javier Rodríguez Jiménez